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MN GENE AND PROTEIN

This application is a continuation of U.S. Serial No. 08/485,049 (filed June 7, 1995), which issued as U.S. Patent 6,204,370 on March 20, 2001, which is a continuation-
5 in-part of now pending U.S. Serial No. 08/260,190 (filed June 15, 1994), which, in turn, is a continuation-in-part of U.S. Serial No. 08/177,093 (filed December 30, 1993), which issued as U.S. Patent 6,051,226 on April 18, 2000, which is, in turn, a continuation-in-part of U.S. Serial No.
10 07/964,589 (filed October 21, 1992), which issued as U.S. Patent No. 5,387,676 on February 7, 1995. This application declares priority under 35 USC § 120 from those U.S. applications, and also under 35 USC § 119 from the now pending Czechoslovakian patent application PV-709-92 (filed
15 March 11, 1992).

FIELD OF THE INVENTION

The present invention is in the general area of medical genetics and in the fields of biochemical engineering and immunochemistry. More specifically, it
20 relates to the identification of a new gene--the MN gene--a

cellular gene coding for the MN protein. The inventors
hereof found MN proteins to be associated with
tumorigenicity. Evidence indicates that the MN protein
appears to represent a potentially novel type of
5 oncoprotein. Identification of MN antigen as well as
antibodies specific therefor in patient samples provides the
basis for diagnostic/prognostic assays for cancer.

BACKGROUND OF THE INVENTION

A novel quasi-viral agent having rather unusual
10 properties was detected by its capacity to complement
mutants of vesicular stomatitis virus (VSV) with heat-labile
surface G protein in HeLa cells (cell line derived from
human cervical adenocarcinoma), which had been cocultivated
with human breast carcinoma cells. [Zavada et al., Nature
15 New Biol., 240: 124 (1972); Zavada et al., J. Gen. Virol.,
24: 327 (1974); Zavada, J., Arch. Virol., 50: 1 (1976);
Zavada, J., J. Gen. Virol., 63: 15-24 (1982); Zavada and
Zavadova, Arch. Virol., 118: 189 (1991).] The quasi viral
agent was called MaTu as it was presumably derived from a
20 human mammary tumor.

There was significant medical interest in studying
and characterizing MaTu as it appeared to be an entirely new
type of molecular parasite of living cells, and possibly

originated from a human tumor. Described herein is the elucidation of the biological and molecular nature of MaTu which resulted in the discovery of the MN gene and protein. MaTu was found by the inventors to be a two-component
5 system, having an exogenous transmissible component, MX, and an endogenous cellular component, MN. As described herein, the MN component was found to be a cellular gene, showing only very little homology with known DNA sequences. The MN gene was found to be present in the chromosomal DNA of all
10 vertebrates tested, and its expression was found to be strongly correlated with tumorigenicity.

The exogenous MaTu-MX transmissible agent was identified as lymphocytic choriomeningitis virus (LCMV) which persistently infects HeLa cells. The inventors
15 discovered that the MN expression in HeLa cells is positively regulated by cell density, and also its expression level is increased by persistent infection with LCMV.

Research results provided herein show that cells
20 transfected with MN cDNA undergo changes indicative of malignant transformation.

Further research findings described herein indicate that the disruption of cell cycle control is one of

the mechanisms by which MN may contribute to the complex process of tumor development.

Described herein is the cloning and sequencing of the MN gene and the recombinant production of MN proteins.

5 Also described are antibodies prepared against MN proteins/polypeptides. MN proteins/polypeptides can be used in serological assays according to this invention to detect MN-specific antibodies. Further, MN proteins/polypeptides and/or antibodies reactive with MN antigen can be used in
10 immunoassays according to this invention to detect and/or quantitate MN antigen. Such assays may be diagnostic and/or prognostic for neoplastic/pre-neoplastic disease.

SUMMARY OF THE INVENTION

Herein disclosed is the MN gene, a cellular gene
15 which is the endogenous component of the MaTu agent. A full-length cDNA sequence for the MN gene is shown in Figures 1A-1C [SEQ. ID. NO.: 1]. Figures 15A-15F provide a complete genomic sequence for MN [SEQ. ID. NO.: 5]. Figure
25 provides the sequence for a proposed MN promoter region
20 [SEQ. ID. NO.: 27].

This invention is directed to the MN gene, fragments thereof and the related cDNA which are useful, for example, as follows: 1) to produce MN proteins/

polypeptides by biochemical engineering; 2) to prepare
nucleic acid probes to test for the presence of the MN gene
in cells of a subject; 3) to prepare appropriate polymerase
chain reaction (PCR) primers for use, for example, in PCR-
5 based assays or to produce nucleic acid probes; 4) to
identify MN proteins and polypeptides as well as homologs or
near homologs thereto; 5) to identify various mRNAs
transcribed from MN genes in various tissues and cell lines,
preferably human; and 6) to identify mutations in MN genes.
10 The invention further concerns purified and isolated DNA
molecules comprising the MN gene or fragments thereof, or
the related cDNA or fragments thereof.

Thus, this invention in one aspect concerns
isolated nucleic acid sequences that encode MN proteins or
15 polypeptides wherein the nucleotide sequences for said
nucleic acids are selected from the group consisting of:

- (a) SEQ. ID. NO.: 1;
- (b) nucleotide sequences that hybridize under
stringent conditions to SEQ. ID. NO.: 1 or to its
20 complement;
- (c) nucleotide sequences that differ from SEQ. ID.
NO.: 1 or from the nucleotide sequences of (b) in codon
sequence because of the degeneracy of the genetic code.
Further, such nucleic acid sequences are selected from

nucleotide sequences that but for the degeneracy of the genetic code would hybridize to SEQ. ID. NO.: 1 or to its complement under stringent hybridization conditions.

Further, such isolated nucleic acids that encode
5 MN proteins or polypeptides can also include the MN nucleic acids of the genomic clone shown in Figures 15A-15F, that is, SEQ. ID. NO.: 5, as well as sequences that hybridize to it or its complement under stringent conditions, or would hybridize to SEQ. ID. NO.: 5 or to its complement under
10 such conditions, but for the degeneracy of the genetic code. Degenerate variants of SEQ. ID. NOS.: 1 and 5 are within the scope of the invention.

Further, this invention concerns nucleic acid probes which are fragments of the isolated nucleic acids
15 that encode MN proteins or polypeptides as described above. Preferably said nucleic acid probes are comprised of at least 29 nucleotides, more preferably of at least 50 nucleotides, still more preferably at least 100 nucleotides, and even more preferably at least 150 nucleotides.

20 Still further, this invention is directed to isolated nucleic acids selected from the group consisting of:

(a) a nucleic acid having the nucleotide sequence shown in Figures 15A-15F [SEQ. ID. NO.: 5] and its complement;

5 (b) nucleic acids that hybridize under standard stringent hybridization conditions to the nucleic acids of (a); and

(c) nucleic acids that differ from the nucleic acids of (a) and (b) in codon sequence because of the degeneracy of the genetic code. The invention also concerns
10 nucleic acids that but for the degeneracy of the genetic code would hybridize to the nucleic acids of (a) under standard stringent hybridization conditions. The nucleic acids of (b) and (c) that hybridize to the coding region of SEQ. ID. NO.: 5 preferably have a length of at least 29
15 nucleotides, whereas the nucleic acids of (b) and (c) that hybridize partially or wholly to the non-coding regions of SEQ. ID. NO.: 5 or its complement are those that function as nucleic acid probes to identify MN nucleic acid sequences. Conventional technology can be used to determine
20 whether the nucleic acids of (b) and (c) or of fragments of SEQ. ID. NO.: 5 are useful to identify MN nucleic acid sequences, for example, as outlined in Benton and Davis, Science, 196: 180 (1977) and Fuscoe et al. Genomics, 5: 100 (1989). In general, the nucleic acids of (b) and (c)

are preferably at least 29 nucleotides, more preferably at least 50 nucleotides, still more preferably at least 100 nucleotides, and even more preferably at least 150 nucleotides. An exemplary and preferred nucleic acid probe is SEQ. ID. NO.: 55 (a 470 bp probe useful in RNase protection assays).

Test kits of this invention can comprise the nucleic acid probes of the invention which are useful diagnostically/prognostically for neoplastic and/or pre-neoplastic disease. Preferred test kits comprise means for detecting or measuring the hybridization of said probes to the MN gene or to the mRNA product of the MN gene, such as a visualizing means.

Fragments of the isolated nucleic acids of the invention, can also be used as PCR primers to amplify segments of MN genes, and may be useful in identifying mutations in MN genes. Typically, said PCR primers are oligonucleotides, preferably at least 16 nucleotides, but they may be considerably longer. Exemplary primers may be from about 16 nucleotides to about 50 nucleotides, preferably from about 19 nucleotides to about 45 nucleotides.

This invention also concerns nucleic acids which encode MN proteins or polypeptides that are specifically

bound by monoclonal antibodies designated M75 that are produced by the hybridoma VU-M75 deposited at the American Type Culture Collection (ATCC) at 10801 University Blvd., Manassas, Virginia 20110-2209 (USA) under ATCC No. HB 11128, and/or by monoclonal antibodies designated MN12 produced by the hybridoma MN 12.2.2 deposited at the ATCC under ATCC No. HB 11647.

The invention further concerns the discovery of a hitherto unknown protein--MN, encoded by the MN gene. The expression of MN proteins is inducible by growing cells in dense cultures, and such expression was discovered to be associated with tumorigenic cells.

MN proteins were found to be produced by some human tumor cell lines in vitro, for example, by HeLa (cervical carcinoma), T24 (bladder carcinoma) and T47D (mammary carcinoma) and SK-Mel 1477 (melanoma) cell lines, by tumorigenic hybrid cells and by cells of some human cancers in vivo, for example, by cells of uterine cervical, ovarian and endometrial carcinomas as well as cells of some benign neoplasias such as mammary papillomas. MN proteins were not found in non-tumorigenic hybrid cells, and are generally not found in the cells of normal tissues, although they have been found in a few normal tissues, most notably and abundantly in normal stomach tissues. MN antigen was

found by immunohistochemical staining to be prevalent in tumor cells and to be present sometimes in morphologically normal appearing areas of tissue specimens exhibiting dysplasia and/or malignancy. Thus, the MN gene is strongly
5 correlated with tumorigenesis and is considered to be a putative oncogene.

In HeLa and in tumorigenic HeLa x fibroblast hybrid (H/F-T) cells, MN protein is manifested as a "twin" protein p54/58N; it is glycosylated and forms disulfide-
10 linked oligomers. As determined by electrophoresis upon reducing gels, MN proteins have molecular weights in the range of from about 40 kd to about 70 kd, preferably from about 45 kd to about 65 kd, more preferably from about 48 kd to about 58 kd. Upon non-reducing gels, MN proteins in the
15 form of oligomers have molecular weights in the range of from about 145 kd to about 160 kd, preferably from about 150 to about 155 kd, still more preferably from about 152 to about 154 kd. A predicted amino acid sequence for a preferred MN protein of this invention is shown in Figures
20 1A-1C [SEQ. ID. NO. 2].

The discovery of the MN gene and protein and thus, of substantially complementary MN genes and proteins encoded thereby, led to the finding that the expression of MN proteins was associated with tumorigenicity. That finding

resulted in the creation of methods that are diagnostic/
prognostic for cancer and precancerous conditions. Methods
and compositions are provided for identifying the onset and
presence of neoplastic disease by detecting and/or
5 quantitating MN antigen in patient samples, including tissue
sections and smears, cell and tissue extracts from
vertebrates, preferably mammals and more preferably humans.
Such MN antigen may also be found in body fluids.

MN proteins and genes are of use in research
10 concerning the molecular mechanisms of oncogenesis, in
cancer diagnostics/prognostics, and may be of use in cancer
immunotherapy. The present invention is useful for
detecting a wide variety of neoplastic and/or pre-neoplastic
diseases. Exemplary neoplastic diseases include carcinomas,
15 such as mammary, bladder, ovarian, uterine, cervical,
endometrial, squamous cell and adenosquamous carcinomas; and
head and neck cancers; mesodermal tumors, such as
neuroblastomas and retinoblastomas; sarcomas, such as
osteosarcomas and Ewing's sarcoma; and melanomas. Of
20 particular interest are head and neck cancers, gynecologic
cancers including ovarian, cervical, vaginal, endometrial
and vulval cancers; gastrointestinal cancer, such as,
stomach, colon and esophageal cancers; urinary tract cancer,
such as, bladder and kidney cancers; skin cancer; liver

cancer; prostate cancer; lung cancer; and breast cancer. Of
still further particular interest are gynecologic cancers;
breast cancer; urinary tract cancers, especially bladder
cancer; lung cancer; and liver cancer. Even further of
5 particular interest are gynecologic cancers and breast
cancer. Gynecologic cancers of particular interest are
carcinomas of the uterine cervix, endometrium and ovaries;
more particularly such gynecologic cancers include cervical
squamous cell carcinomas, adenosquamous carcinomas,
10 adenocarcinomas as well as gynecologic precancerous
conditions, such as metaplastic cervical tissues and
condylomas.

The invention further relates to the biochemical
engineering of the MN gene, fragments thereof or related
15 cDNA. For example, said gene or a fragment thereof or
related cDNA can be inserted into a suitable expression
vector; host cells can be transformed with such an
expression vector; and an MN protein/polypeptide, preferably
an MN protein, is expressed therein. Such a recombinant
20 protein or polypeptide can be glycosylated or
nonglycosylated, preferably glycosylated, and can be
purified to substantial purity. The invention further
concerns MN proteins/polypeptides which are synthetically or
otherwise biologically prepared.

Said MN proteins/polypeptides can be used in assays to detect MN antigen in patient samples and in serological assays to test for MN-specific antibodies. MN proteins/polypeptides of this invention are serologically active, immunogenic and/or antigenic. They can further be used as immunogens to produce MN-specific antibodies, polyclonal and/or monoclonal, as well as an immune T-cell response.

The invention further is directed to MN-specific antibodies, which can be used diagnostically/prognostically and may be used therapeutically. Preferred according to this invention are MN-specific antibodies reactive with the epitopes represented respectively by the amino acid sequences of the MN protein shown in Figures 1A-1C as follows: from AA 62 to AA 67 [SEQ. ID. NO.: 10]; from AA 55 to AA 60 [SEQ. ID. NO.: 11]; from AA 127 to AA 147 [SEQ. ID. NO.: 12]; from AA 36 to AA 51 [SEQ. ID. NO.: 13]; from AA 68 to AA 91 [SEQ. ID. NO.: 14]; from AA 279 to AA 291 [SEQ. ID. NO.: 15]; and from AA 435 to AA 450 [SEQ. ID. NO.: 16]. More preferred are antibodies reactive with epitopes represented by SEQ. ID. NOS.: 10, 11 and 12. Still more preferred are antibodies reactive with the epitopes represented by SEQ. ID NOS: 10 and 11, as for example, respectively Mabs M75 and MN12. Most preferred are

monoclonal antibodies reactive with the epitope represented by SEQ. ID. NO.: 10.

Also preferred according to this invention are antibodies prepared against recombinantly produced MN proteins as, for example, GEX-3X-MN, MN 20-19, MN-Fc and MN-PA. Also preferred are MN-specific antibodies prepared against glycosylated MN proteins, such as, MN 20-19 expressed in baculovirus infected Sf9 cells.

A hybridoma that produces a representative MN-specific antibody, the monoclonal antibody M75 (Mab M75), was deposited at the ATCC under Number HB 11128 as indicated above. The M75 antibody was used to discover and identify the MN protein and can be used to identify readily MN antigen in Western blots, in radioimmunoassays and immunohistochemically, for example, in tissue samples that are fresh, frozen, or formalin-, alcohol-, acetone- or otherwise fixed and/or paraffin-embedded and deparaffinized. Another representative MN-specific antibody, Mab MN12, is secreted by the hybridoma MN 12.2.2, which was deposited at the ATCC under the designation HB 11647.

MN-specific antibodies can be used, for example, in laboratory diagnostics, using immunofluorescence microscopy or immunohistochemical staining; as a component in immunoassays for detecting and/or quantitating MN antigen

in, for example, clinical samples; as probes for immunoblotting to detect MN antigen; in immunoelectron microscopy with colloid gold beads for localization of MN proteins and/or polypeptides in cells; and in genetic engineering for cloning the MN gene or fragments thereof, or
5 related cDNA. Such MN-specific antibodies can be used as components of diagnostic/ prognostic kits, for example, for in vitro use on histological sections; such antibodies can also and used for in vivo diagnostics/prognostics, for
10 example, such antibodies can be labeled appropriately, as with a suitable radioactive isotope, and used in vivo to locate metastases by scintigraphy. Further such antibodies may be used in vivo therapeutically to treat cancer patients with or without toxic and/or cytostatic agents attached
15 thereto. Further, such antibodies can be used in vivo to detect the presence of neoplastic and/or pre-neoplastic disease. Still further, such antibodies can be used to affinity purify MN proteins and polypeptides.

This invention also concerns recombinant DNA
20 molecules comprising a DNA sequence that encodes for an MN protein or polypeptide, and also recombinant DNA molecules that encode not only for an MN protein or polypeptide but also for an amino acid sequence of a non-MN protein or polypeptide. Said non-MN protein or polypeptide may

preferably be nonimmunogenic to humans and not typically reactive to antibodies in human body fluids. Examples of such a DNA sequence is the alpha-peptide coding region of beta-galactosidase and a sequence coding for glutathione S-transferase or a fragment thereof. However, in some instances, a non-MN protein or polypeptide that is serologically active, immunogenic and/or antigenic may be preferred as a fusion partner to a MN antigen. Further, claimed herein are such recombinant fusion proteins/
10 polypeptides which are substantially pure and non-naturally occurring. Exemplary fusion proteins of this invention are GEX-3X-MN, MN-Fc and MN-PA, described infra.

This invention also concerns methods of treating neoplastic disease and/or pre-neoplastic disease comprising
15 inhibiting the expression of MN genes by administering antisense nucleic acid sequences that are substantially complementary to mRNA transcribed from MN genes. Said antisense nucleic acid sequences are those that hybridize to such mRNA under stringent hybridization conditions.
20 Preferred are antisense nucleic acid sequences that are substantially complementary to sequences at the 5' end of the MN cDNA sequence shown in Figures 1A-1C. Preferably said antisense nucleic acid sequences are oligonucleotides.

This invention also concerns vaccines comprising an immunogenic amount of one or more substantially pure MN proteins and/or polypeptides dispersed in a physiologically acceptable, nontoxic vehicle, which amount is effective to
5 immunize a vertebrate, preferably a mammal, more preferably a human, against a neoplastic disease associated with the expression of MN proteins. Said proteins can be recombinantly, synthetically or otherwise biologically produced. Recombinant MN proteins include GEX-3X-MN and MN
10 20-19. A particular use of said vaccine would be to prevent recidivism and/or metastasis. For example, it could be administered to a patient who has had an MN-carrying tumor surgically removed, to prevent recurrence of the tumor.

The immunoassays of this invention can be embodied
15 in test kits which comprise MN proteins/polypeptides and/or MN-specific antibodies. Such test kits can be in solid phase formats, but are not limited thereto, and can also be in liquid phase format, and can be based on immunohistochemical assays, ELISAs, particle assays,
20 radiometric or fluorometric assays either unamplified or amplified, using, for example, avidin/biotin technology.

Abbreviations

The following abbreviations are used herein:

| | | | |
|----|-------------------|---|-----------------------------------|
| | AA | - | amino acid |
| | ATCC | - | American Type Culture Collection |
| 5 | bp | - | base pairs |
| | BLV | - | bovine leukemia virus |
| | BSA | - | bovine serum albumin |
| | BRL | - | Bethesda Research Laboratories |
| | CA | - | carbonic anhydrase |
| 10 | CAT | - | chloramphenicol acetyltransferase |
| | Ci | - | curie |
| | cm | - | centimeter |
| | CMV | - | cytomegalovirus |
| | cpm | - | counts per minute |
| 15 | C-terminus | - | carboxyl-terminus |
| | °C | - | degrees centigrade |
| | DAB | - | diaminobenzidine |
| | dH ₂ O | - | deionized water |
| | DEAE | - | diethylaminoethyl |
| 20 | DMEM | - | Dulbecco modified Eagle medium |
| | DTT | - | dithiothreitol |
| | EDTA | - | ethylenediaminetetracetate |
| | EIA | - | enzyme immunoassay |
| | ELISA | - | enzyme-linked immunosorbent assay |

| | | | |
|----|-------------------------------|---|--|
| | EtOH | - | ethanol |
| | F | - | fibroblasts |
| | FCS | - | fetal calf serum |
| | FIBR | - | fibroblasts |
| 5 | FITC | - | fluorescein isothiocyanate |
| | GEX-3X-MN | - | fusion protein MN glutathione S-transferase |
| | H | - | HeLa cells |
| | H ₂ O ₂ | - | hydrogen peroxide |
| | HCA | - | Hydrophobic Cluster Analysis |
| 10 | HEF | - | human embryo fibroblasts |
| | HeLa K | - | standard type of HeLa cells |
| | HeLa S | - | Stanbridge's mutant HeLa D98/AH.2 |
| | H/F-T | - | hybrid HeLa fibroblast cells that are tumorigenic; derived from HeLa D98/AH.2 |
| 15 | H/F-N | - | hybrid HeLa fibroblast cells that are nontumorigenic; derived from HeLa D98/AH.2 |
| | HGPRT ⁻ | - | hypoxanthine guanine phosphoribosyl transferase-deficient |
| | HLH | - | helix-loop-helix |
| 20 | HRP | - | horseradish peroxidase |
| | Inr | - | initiator |
| | IPTG | - | isopropyl-beta-D-thiogalacto-pyranoside |
| | kb | - | kilobase |
| | kbp | - | kilobase pairs |
| 25 | kd | - | kilodaltons |

| | | | |
|----|------------|---|---------------------------------------|
| | KPL | - | Kirkegaard & Perry Laboratories, Inc. |
| | LCMV | - | lymphocytic choriomeningitis virus |
| | LTR | - | long terminal repeat |
| | M | - | molar |
| 5 | mA | - | milliampere |
| | MAb | - | monoclonal antibody |
| | ME | - | mercaptoethanol |
| | MEM | - | minimal essential medium |
| | min. | - | minute(s) |
| 10 | mg | - | milligram |
| | ml | - | milliliter |
| | mM | - | millimolar |
| | MMC | - | mitomycin C |
| | MLV | - | murine leukemia virus |
| 15 | MTV | - | mammary tumor virus |
| | N | - | normal concentration |
| | ng | - | nanogram |
| | NGS | - | normal goat serum |
| | nt | - | nucleotide |
| 20 | N-terminus | - | amino-terminus |
| | ODN | - | oligodeoxynucleotide |
| | ORF | - | open reading frame |
| | PA | - | Protein A |
| | PAGE | - | polyacrylamide gel electrophoresis |

| | | | |
|----|----------|---|---|
| | PBS | - | phosphate buffered saline |
| | PCR | - | polymerase chain reaction |
| | PEST | - | combination of one-letter abbreviations for proline, glutamic acid, serine, threonine |
| 5 | pI | - | isoelectric point |
| | PMA | - | phorbol 12-myristate 13-acetate |
| | Py | - | pyrimidine |
| | RIP | - | radioimmunoprecipitation |
| | RIPA | - | radioimmunoprecipitation assay |
| 10 | RNP | - | RNase protection assay |
| | SAC | - | protein A- <u>Staphylococcus aureus</u> cells |
| | SDRE | - | serum dose response element |
| | SDS | - | sodium dodecyl sulfate |
| 15 | SDS-PAGE | - | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| | SINE | - | short interspersed repeated sequence |
| | SP-RIA | - | solid-phase radioimmunoassay |
| | SSDS | - | synthetic splice donor site |
| 20 | SSPE | - | NaCl (0.18 M), sodium phosphate (0.01 M), EDTA (0.001 M) |
| | TBE | - | Tris-borate/EDTA electrophoresis buffer |
| | TCA | - | trichloroacetic acid |
| | TC media | - | tissue culture media |
| | TMB | - | tetramethylbenzidine |
| 25 | Tris | - | tris (hydroxymethyl) aminomethane |

| | | | |
|---|----------|---|----------------------------------|
| | μ Ci | - | microcurie |
| | μ g | - | microgram |
| | μ l | - | microliter |
| | μ M | - | micromolar |
| 5 | VSV | - | vesicular stomatitis virus |
| | X-MLV | - | xenotropic murine leukemia virus |

Cell Lines

The following cell lines were used in the experiments herein described:

| | | | |
|----|-----------------------------|----|--|
| 10 | HeLa K | -- | standard type of HeLa cells; aneuploid, epithelial-like cell line isolated from a human cervical adenocarcinoma [Gey et al., <u>Cancer Res.</u> , 12: 264 (1952); Jones et al., <u>Obstet. Gynecol.</u> , 38: 945-949 (1971)] |
| 15 | | | obtained from Professor B. Korych, [Institute of Medical Microbiology and Immunology, Charles University; Prague, Czech Republic] |
| 20 | HeLa D98/AH.2 (also HeLa S) | -- | Mutant HeLa clone that is hypoxanthine guanine phosphoribosyl transferase-deficient (HGPRT ⁻) kindly provided by Eric J. Stanbridge [Department of Microbiology, College of Medicine, University of California, Irvine, CA (USA)] and reported |
| 25 | | | in Stanbridge et al., <u>Science</u> , 215: 252-259 (15 Jan. 1982); parent of hybrid cells H/F-N and H/F-T, also obtained from E.J. Stanbridge. |
| 30 | NIH-3T3 | -- | murine fibroblast cell line reported in Aaronson, <u>Science</u> , 237: 178 (1987). |
| | T47D | -- | cell line derived from a human mammary carcinoma [Keydar et al., <u>Eur. J. Cancer</u> , 15: 659-670 (1979)]; kindly provided by J. |

Keydar [Haddasah Medical School; Jerusalem, Israel]

- 5 T24 -- cell line from urinary bladder carcinoma [Bubenik et al., Int. J. Cancer, 11: 765-773 (1973)] kindly provided by J. Bubenik [Institute of Molecular Genetics, Czechoslovak Academy of Sciences; Prague, Czech Republic]
- 10 HMB2 -- cell line from melanoma [Svec et al., Neoplasma, 35: 665-681 (1988)]
- HEF -- human embryo fibroblasts [Zavada et al., Nature New Biology, 240: 124-125 (1972)]
- 15 SIRC -- cell line from rabbit cornea (control and X-MLV-infected) [Zavada et al., Virology, 82: 221-231 (1977)]
- Vero cells -- African green monkey cell line [Zavada et al. (1977)]
- 20 myeloma cell line NS-0 -- myeloma cell line used as a fusion parent in production of monoclonal antibodies [Galfre and Milstein, Methods Enzymol., 73: 3-46 (1981)]
- 25 SK-Mel 1477 -- human melanoma cell line kindly provided by K.E. Hellstrom [Division of Tumor Immunology, Fred Hutchins Cancer Research Center; Seattle, Washington (USA)]
- 30 XC -- cells derived from a rat rhabdomyosarcoma induced with Rous sarcoma virus-induced rat sarcoma [Svoboda, J., Natl. Cancer Center Institute Monograph No. 17, IN: "International Conference on Avian Tumor Viruses" (J.W. Beard ed.), pp. 277-298 (1964)], kindly provided by Jan Svoboda [Institute of Molecular Genetics, Czechoslovak Academy of Sciences; Prague, Czech Republic]; and
- 35 Rat 2-Tk -- a thymidine kinase deficient cell line, kindly provided by L. Kutinova [Institute

of Sera and Vaccines; Prague, Czech Republic]

| | | | |
|----|------|----|---|
| | CGL1 | -- | H/F-N hybrid cells (HeLa D98/AH.2 derivative) |
| 5 | CGL2 | -- | H/F-N hybrid cells (HeLa D98/AH.2 derivative) |
| | CGL3 | -- | H/F-T hybrid cells (HeLa D98/AH.2 derivative) |
| 10 | CGL4 | -- | H/F-T hybrid cells (HeLa D98/Ah.2 derivative) |

Nucleotide and Amino Acid Sequence Symbols

The following symbols are used to represent nucleotides herein:

| | <u>Base Symbol</u> | <u>Meaning</u> |
|----|------------------------|----------------|
| 15 | A | adenine |
| | C | cytosine |
| | G | guanine |
| | T | thymine |
| | U | uracil |
| 20 | I | inosine |
| | M | A or C |
| | R | A or G |
| | W | A or T/U |
| | S | C or G |
| 25 | Y | C or T/U |

| | | |
|---|-----|--------------------|
| | K | G or T/U |
| | V | A or C or G |
| | H | A or C or T/U |
| | D | A or G or T/U |
| 5 | B | C or G or T/U |
| | N/X | A or C or G or T/U |

There are twenty main amino acids, each of which is specified by a different arrangement of three adjacent nucleotides (triplet code or codon), and which are linked together in a specific order to form a characteristic protein. A three-letter or one-letter convention is used herein to identify said amino acids, as, for example, in Figures 1A-1C as follows:

| | | | |
|----|------------------------|-----------------------|-----------------------|
| 15 | <u>Amino acid name</u> | <u>3 Ltr. Abbrev.</u> | <u>1 Ltr. Abbrev.</u> |
| | Alanine | Ala | A |
| | Arginine | Arg | R |
| | Asparagine | Asn | N |
| | Aspartic Acid | Asp | D |
| 20 | Cysteine | Cys | C |
| | Glutamic Acid | Glu | E |
| | Glutamine | Gln | Q |
| | Glycine | Gly | G |
| | Histidine | His | H |

| | | | |
|----|------------------|-----|---|
| | Isoleucine | Ile | I |
| | Leucine | Leu | L |
| | Lysine | Lys | K |
| | Methionine | Met | M |
| 5 | Phenylalanine | Phe | F |
| | Proline | Pro | P |
| | Serine | Ser | S |
| | Threonine | Thr | T |
| | Tryptophan | Trp | W |
| 10 | Tyrosine | Tyr | Y |
| | Valine | Val | V |
| | Unknown or other | | X |

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1C provide the nucleotide sequence for
15 a full-length MN cDNA [SEQ. ID. NO.: 1] clone isolated as
described herein. Figures 1A-1C also set forth the
predicted amino acid sequence [SEQ. ID. NO.: 2] encoded by
the cDNA.

Figure 2 provides SDS-PAGE and immunoblotting
20 analyses of recombinant MN protein expressed from a pGEX-3X
bacterial expression vector. Two parallel samples of
purified recombinant MN protein (twenty μ g in each sample)
were separated by SDS-PAGE on a 10% gel. One sample (A in

Figure 2) was stained with Coomassie brilliant blue; whereas the other sample (B) was blotted onto a Hybond C membrane [Amersham; Aylesbury, Bucks, England]. The blot was developed by autoradiography with ^{125}I -labeled Mab M75.

5 Figure 3 illustrates inhibition of p54/58 expression by antisense oligodeoxynucleotides (ODNs). HeLa cells cultured in overcrowded conditions were incubated with (A) 29-mer ODNI [SEQ. ID. NO.: 3]; (B) 19-mer ODN2 [SEQ. ID. NO.: 4]; (C) both ODNI and ODN2; and (D) without ODNs. 10 Example 10 provides details of the procedures used.

 Figure 4 shows the results of Northern blotting of MN mRNA in human cell lines. Total RNA was prepared from the following cell lines: HeLa cells growing in dense (A) and sparse (B) culture; (C) H/F-N; (D) and (E) H/F-T; and 15 (F) human embryo fibroblasts. Example 11 details the procedure and results.

 Figure 5 illustrates the detection of the MN gene in genomic DNAs by Southern blotting. Chromosomal DNA digested by PstI was as follows: (A) chicken; (B) bat; (C) 20 rat; (D) mouse; (E) feline; (F) pig; (G) sheep; (H) bovine; (I) monkey; and (J) human HeLa cells. The procedures used are detailed in Example 12.

 Figure 6 graphically illustrates the expression of MN- and MX-specific proteins in human fibroblasts (F), in

HeLa cells (H) and in H/F-N and H/F-T hybrid cells and contrasts the expression in MX-infected and MX-uninfected cells. Example 5 details the procedures and results.

Figure 7 (discussed in Example 5) provides
5 immunoblots of MN proteins in fibroblasts (FIBR) and in HeLa K, HeLa S, H/F-N and H/F-T hybrid cells.

Figure 8 (discussed in Example 6) shows
immunoblots of MN proteins in cell culture extracts prepared from the following: (A) MX-infected HeLa cells; (B) human
10 fibroblasts; (C) T24; (D) T47D; (E) SK-Mel 1477; and (F) HeLa cells uninfected with MX. The symbols +ME and O ME indicate that the proteins were separated by PAGE after heating in a sample buffer, with and without 3% mercaptoethanol (ME), respectively.

15 Figure 9 (discussed in Example 6) provides immunoblots of MN proteins from human tissue extracts. The extracts were prepared from the following: (A) MX-infected HeLa cells; (B) full-term placenta; (C) corpus uteri; (D, M) adenocarcinoma endometrii; (E, N) carcinoma ovarii; (F, G)
20 trophoblasts; (H) normal ovary; (I) myoma uteri; (J) mammary papilloma; (K) normal mammary gland; (L) hyperplastic endometrium; (O) cervical carcinoma; and (P) melanoma.

Figure 10 (discussed in Example 7) provides immunoblots of MN proteins from (A) MX-infected HeLa cells

and from (B) Rat2-Tk cells. (+ME and 0 ME have the same meanings as explained in the legend to Figure 8.)

Figures 11A and 11B (discussed in Example 8) graphically illustrate the results from
5 radioimmunoprecipitation experiments with ^{125}I -GEX-3X-MN protein and different antibodies. The radioactive protein (15×10^3 cpm/tube) was precipitated with ascitic fluid or sera and SAC as follows: (A) ascites with MAb M75; (B) rabbit anti-MaTu serum; (C) normal rabbit serum; (D) human
10 serum L8; (E) human serum KH; and (F) human serum M7.

Figure 12 (discussed in Example 8) shows the results from radioimmunoassays for MN antigen. Ascitic fluid (dilution precipitating 50% radioactivity) was allowed to react for 2 hours with (A) "cold" (unlabeled) protein
15 GEX-3X-MN, or with extracts from cells as follows: (B) HeLa + MX; (C) Rat-2Tk; (D) HeLa; (E) rat XC; (F) T24; and (G) HEF. Subsequently ^{125}I -labeled GEX-3X-MN protein (25×10^3 cpm/tube) was added and incubated for an additional 2 hours. Finally, the radioactivity to MAb M75 was adsorbed to SAC
20 and measured.

Figure 13 (discussed in Example 9) provides results of immunoelectron and scanning microscopy of MX-uninfected (control) and MX-infected HeLa cells. Panels A-D show ultrathin sections of cells stained with MAb M75 and

immunogold; Panels E and F are scanning electron micrographs of cells wherein no immunogold was used. Panels E and F both show a terminal phase of cell division. Panels A and E are of control HeLa cells; panels B, C, D and F are of MX-
5 infected HeLa cells. The cells shown in Panels A, B and C were fixed and treated with M75 and immunogold before they were embedded and sectioned. Such a procedure allows for immunogold decoration only of cell surface antigens. The cells in Panel D were treated with M75 and immunogold only
10 once they had been embedded and sectioned, and thus antigens inside the cells could also be decorated.

Figure 14 compares the results of immunizing baby rats to XC tumor cells with rat serum prepared against the fusion protein MN glutathione S-transferase (GEX-3X-MN) (the
15 IM group) with the results of immunizing baby rats with control rat sera (the C group). Each point on the graph represents the tumor weight of a tumor from one rat. Example 14 details those experiments.

Figures 15A-15F provide a 10,898 bp complete
20 genomic sequence of MN [SEQ. ID. NO.: 5]. The base count is as follows: 2654 A; 2739 C; 2645 G; and 2859 T. The 11 exons are shown in capital letters.

Figure 16 is a restriction map of the full-length MN cDNA. The open reading frame is shown as an open box.

The thick lines below the restriction map illustrate the sizes and positions of two overlapping cDNA clones. The horizontal arrows indicate the positions of primers R1 [SEQ. ID. NO.: 7] and R2 [SEQ. ID. NO.: 8] used for the 5' end RACE. Relevant restriction sites are BamHI (B), EcoRV (V), EcoRI (E), PstI (Ps), PvuII (Pv).

Figure 17 shows a restriction analysis of the MN gene. Genomic DNA from HeLa cells was cleaved with the following restriction enzymes: EcoRI (1), EcoRV (2), HindIII (3), KpnI (4), NcoI (5), PstI (6), and PvuII (7), and then analyzed by Southern hybridization under stringent conditions using MN cDNA as a probe.

Figure 18 is a mapping of the transcription initiation (a) and termination (b) sites by RNase protection assay. MN-specific protected RNA fragments from CGL3 cells (2), HeLa (3) and HELA persistently infected with LCMV (4) are indicated with arrows. NIH 3T3 cells (1) that do not express MN serve as a negative control.

Figure 19(a) shows an alignment of HCA plots derived from MN, human CA VI (hCA) and CA II (CA2). A one-letter code is used for all amino acids with exception of P (stars), G (diamond-shaped symbol), T and S (open and dotted squares, respectively). Strands D, E, F and G are essential for the structural core of CA. Topologically conserved

hydrophobic amino acids are shaded (in hCA VI and MN).
Ligands of the catalytic zinc ion (His residues) are
indicated by arrowheads.

Figure 19(b) presents a stereoview of the CA II
three-dimensional structure illustrating a superposition of
the complete CA II structure (thin ribbon) with the
structure which is well conserved in MN (open thick ribbon).

Figure 20 schematically represents the 5' MN
genomic region of an MN genomic clone.

Figure 21(a) shows the zinc-binding activity of MN
protein extracted from HeLa cells persistently infected with
LCMV. Samples were concentrated by immunoprecipitation with
Mab M75 before loading (A), and after elution from ZnCl_2 -
saturated (B) or ZnCl_2 -free Fast-Flow chelating Sepharase
column (c). Immunoprecipitates were analyzed by Western
blotting using iodinated M75 antibody.

Figure 21(b) shows MN protein binding to DNA-
cellulose. Proteins extracted from LCMV-infected HeLa cells
were incubated with DNA-cellulose (A). Proteins that bound
to DNA-cellulose in the presence of ZnCl_2 and absence of DTT
(B), in the presence of both ZnCl_2 and DTT (C), and in the
absence of both ZnCl_2 and DTT (D) were eluted, and all
samples were analyzed as above.

Figure 21(c) shows the results of endoglycosidase H and F digestion. MN protein immunoprecipitated with Mab M75 was treated with Endo F (F) and Endo H (H). Treated (+) and control samples (-) were analyzed by Western blotting as above.

Figure 22 shows the morphology and growth kinetics of control (a, c, e and g) and MN-expressing (b, d, f and h) NIH 3T3 cells. The micrographs are of methanol fixed and Giemsa stained cells at a magnification X 100. Cells were grown to confluency (a, b), or as individual colonies in Petri dishes (c, d) and in soft agar (e, f). The (g) and (h) graphs provide growth curves of cells cultured in DMEM medium containing respectively, 10% and 1% FCS. The mean values of triplicate determinations were plotted against time.

Figure 23A-1 to 23C illustrate flow cytometric analyses of asynchronous cell populations of control and MN cDNA-transfected NIH 3T3 cells.

Figure 24 is a map of the human MN gene. The numbered black boxes represent exons. The box designated LTR denotes a region of homology to HERV-K LTR. The empty boxes are Alu-related sequences.

Figure 25 is a nucleotide sequence for the proposed promoter of the human MN gene [SEQ. ID. No.: 27].

The nucleotides are numbered from the transcription initiation site according to RNase protection assay. Potential regulatory elements are overlined. Transcription start sites are indicated by asterisks (RNase protection) and dots (RACE). The sequence of the 1st exon begins under the asterisks.

Figure 26 shows a CpG-rich island of a human MN gene. Each vertical line on the scale represents a CpG doublet (upper map) or a GpC doublet (lower map). CpG is 4-5 fold deficient in comparison to GpC, except the island region where the frequency increases about 5 time. CpG and GpC frequencies are roughly equal in the island region.

Figure 27 provides a schematic of the alignment of MN genomic clones according to their position related to the transcription initiation site. All the genomic fragments except Bd3 were isolated from a lambda FIX III genomic library derived from HeLa cells. Clone Bd3 was derived from a human fetal brain library.

Figure 28 shows the construction and cloning of a series of 5' deletion mutants of MN's putative promoter region linked to the bacterial CAT gene.

Figure 29 outlines the structure of MN promoter-CAT constructs.

DETAILED DESCRIPTION

As demonstrated herein MaTu was found to be a two-component system. One part of the complex, exogenous MX, is transmissible, and is manifested by a protein, p58X, which
5 is a cytoplasmic antigen which reacts with some natural sera, of humans and of various animals. The other component, MN, is endogenous to human cells. The level of MN expression has been found to be considerably increased in the presence of the MaTu-MX transmissible agent, which has
10 been now identified as lymphocytic choriomeningitis virus (LCMV) which persistently infects HeLa cells.

MN is a cellular gene, showing only very little homology with known DNA sequences. It is rather conservative and is present as a single copy gene in the
15 chromosomal DNA of various vertebrates. The MN gene is shown herein to be organized into 11 exons and 10 introns. Described herein is the cloning and sequencing of the MN cDNA and genomic sequences, and the genetic engineering of MN proteins -- such as the GEX-3X-MN, MN-PA, MN-Fc and MN
20 20-19 proteins. The recombinant MN proteins can be conveniently purified by affinity chromatography.

MN is manifested in HeLa cells by a twin protein, p54/58N, that is localized on the cell surface and in the nucleus. Immunoblots using a monoclonal antibody reactive

with p54/58N (MAb M75) revealed two bands at 54 kd and 58 kd. Those two bands may correspond to one type of protein that differs by glycosylation pattern or by how it is processed. (Both p54N and p58N are glycosylated with
5 oligosaccharidic residues containing mannose, but only p58N also contains glucosamine.) Herein, the phrase "twin protein" indicates p54/58N.

MN is absent in rapidly growing, sparse cultures of HeLa, but is inducible either by keeping the cells in
10 dense cultures or, more efficiently, by infecting them with MX (LCMV). Those inducing factors are synergistic. p54/58N and not p58X is associated with virions of vesicular stomatitis virus (VSV), reproduced in MaTu-infected HeLa. Whereas the twin protein p54/58N is glycosylated and forms
15 oligomers linked by disulfidic bonds, p58X is not glycosylated and does not form S-S-linked oligomers.

VSV assembles p54/58N into virions in HeLa cells, indicating that the twin protein is responsible for complementation of VSV G-protein mutants and for formation
20 of VSV(MaTu) pseudotypes. As only enveloped viruses provide surface glycoproteins for the formation of infectious, functioning pseudotypes, which can perform such specific functions as adsorption and penetration of virions into cells [Zavada, J., J. Gen. Virol., 63: 15-24 (1982)], that

observation implies that the MN gene behaves as a quasi-viral sequence.

The surface proteins of enveloped viruses, which participate in the formation of VSV pseudotypes, are glycosylated as is the MN twin protein, p54/58N. MN proteins also resemble viral glycoproteins in the formation of oligomers (preferably tri- or tetramers); such oligomerization, although not necessarily involving S-S bonds (disulfidic bonds), is essential for the assembly of virions [Kreis and Lodish, Cell, 46: 929-937 (1986)]. The disulfidic bonds can be disrupted by reduction with 2-mercaptoethanol.

As reported in Pastorekova et al., Virology, 187: 620-626 (1992), after reduction with mercaptoethanol, p54/58N from cell extracts or from VSV looks very similar on immunoblot. Without reduction, in cell extracts, it gives several bands around 150 kd, suggesting that the cells may contain several different oligomers (probably with a different p54:p58 ratio), but VSV selectively assembles only one of them, with a molecular weight of about 153 kd. That oligomer might be a trimer, or rather a tetramer, consisting of 54 kd and 58 kd proteins. The equimolar ratio of p54:p58 in VSV virions is indicated by approximately the same

strength of 54 kd and 58 kd bands in a VSV sample analyzed under reducing conditions.

The expression of MN proteins appears to be diagnostic/prognostic for neoplastic disease. The MN twin
5 protein, p54/58N, was found to be expressed in HeLa cells and in Stanbridge's tumorigenic (H/F-T) hybrid cells [Stanbridge et al., Somatic Cell Genet, 7: 699-712 (1981); and Stanbridge et al., Science, 215: 252-259 (1982)] but not in fibroblasts or in non-tumorigenic (H/F-N) hybrid
10 cells [Stanbridge et al., id.]. In early studies, MN proteins were found in immunoblots prepared from human ovarian, endometrial and uterine cervical carcinomas, and in some benign neoplasias (as mammary papilloma) but not from normal ovarian, endometrial, uterine or placental tissues.
15 Example 13 details further research on MN gene expression wherein MN antigen, as detected by immunohistochemical staining, was found to be prevalent in tumor cells of a number of cancers, including cervical, bladder, head and neck, and renal cell carcinomas among others. Further, the
20 immunohistochemical staining experiments of Example 13 show that among normal tissues tested, only normal stomach tissues showed routinely and extensively the presence of MN antigen. MN antigen is further shown herein to be present

sometimes in morphologically normal-appearing areas of tissue specimens exhibiting dysplasia and/or malignancy.

In HeLa cells infected with MX, observed were conspicuous ultrastructural alterations, that is, the formation of abundant filaments on cell surfaces and the amplification of mitochondria. Using an immunogold technique, p54/58N was visualized on the surface filaments and in the nucleus, particularly in the nucleoli. Thus MN proteins appear to be strongly correlated with tumorigenicity, and do not appear to be produced in general by normal non-tumor cells.

Examples herein show that MX and MN are two different entities, that can exist independently of each other. MX (LCMV) as an exogenous, transmissible agent can multiply in fibroblasts and in H/F-N hybrid cells which are not expressing MN-related proteins (Figures 6A and 6B). In such cells, MX does not induce the production of MN protein. MN protein can be produced in HeLa and other tumor cells even in the absence of MX as shown in Figures 6-9. However, MX is a potent inducer of MN-related protein in HeLa cells; it increases its production thirty times over the concentration observed in uninfected cells (Figures 7 and 12, Table 2 in Example 8, below).

MN Gene--Cloning and Sequencing

Figures 1A-1C provide the nucleotide sequence for a full-length MN cDNA clone isolated as described below [SEQ. ID. NO.: 1]. Figures 15A-15F provide a complete MN
5 genomic sequence [SEQ. ID. NO.: 5]. Figure 25 shows the nucleotide sequence for a proposed MN promoter [SEQ. ID. NO.: 27].

It is understood that because of the degeneracy of the genetic code, that is, that more than one codon will
10 code for one amino acid [for example, the codons TTA, TTG, CTT, CTC, CTA and CTG each code for the amino acid leucine (leu)], that variations of the nucleotide sequences in, for example, SEQ. ID. NOS.: 1 and 5 wherein one codon is substituted for another, would produce a substantially
15 equivalent protein or polypeptide according to this invention. All such variations in the nucleotide sequences of the MN cDNA and complementary nucleic acid sequences are included within the scope of this invention.

It is further understood that the nucleotide
20 sequences herein described and shown in Figures 1A-1C, 15A-15F and 25, represent only the precise structures of the cDNA, genomic and promoter nucleotide sequences isolated and described herein. It is expected that slightly modified nucleotide sequences will be found or can be modified by

techniques known in the art to code for substantially similar or homologous MN proteins and polypeptides, for example, those having similar epitopes, and such nucleotide sequences and proteins/polypeptides are considered to be
5 equivalents for the purpose of this invention. DNA or RNA having equivalent codons is considered within the scope of the invention, as are synthetic nucleic acid sequences that encode proteins/polypeptides homologous or substantially homologous to MN proteins/polypeptides, as well as those
10 nucleic acid sequences that would hybridize to said exemplary sequences [SEQ. ID. NOS. 1, 5 and 27] under stringent conditions or that but for the degeneracy of the genetic code would hybridize to said cDNA nucleotide sequences under stringent hybridization conditions.
15 Modifications and variations of nucleic acid sequences as indicated herein are considered to result in sequences that are substantially the same as the exemplary MN sequences and fragments thereof.

Partial cDNA clone

20 To find the MN gene, a lambda gt11 cDNA library from MX-infected HeLa cells was prepared. Total RNA from MX-infected HeLa cells was isolated by a guanidinium-thiocyanate-CsCl method [Chirgwin et al., Biochemistry, 18:

5249 (1979)], and the mRNA was affinity separated on oligo dT-cellulose [Ausubel et al., Short Protocols in Molecular Biology, (Greene Publishing Assocs. and Wiley-Interscience; NY, USA, 1989)]. The synthesis of the cDNA and its cloning
5 into lambda gt11 was carried out using kits from Amersham, except that the EcoRI-NotI adaptor was from Stratagene [La Jolla, CA (USA)]. The library was subjected to immunoscreening with Mab M75 in combination with goat anti-mouse antibodies conjugated with alkaline phosphatase. That
10 immunoscreening method is described in Young and Davis, PNAS (USA), 80: 1194-1198 (1983). About 4×10^5 primary plaques on E. coli Y1090 cells, representing about one-half of the whole library, were screened using Hybond N+ membrane [Amersham] saturated with 10 mM IPTG and blocked with 5%
15 FCS. Fusion proteins were detected with Mab M75 in combination with goat anti-mouse antibodies conjugated with alkaline phosphatase. One positive clone was picked.

pBluescript-MN. The positive clone was subcloned into the NotI site of pBluescript KS [Stratagene] thereby
20 creating pBluescript-MN. Two oppositely oriented nested deletions were made using Erase-a-Base™ kit [Promega; Madison, WI (USA)] and sequenced by dideoxy method with a T7 sequencing kit [Pharmacia; Piscataway, NJ (USA)]. The sequencing showed a partial cDNA clone, the insert being

1397 bp long. The sequence comprises a large 1290 bp open reading frame and 107 bp 3' untranslated region containing a polyadenylation signal (AATAAA). Another interesting feature of the sequence is the presence of a region contributing to instability of the mRNA (AUUUA at position 1389) which is characteristic for mRNAs of some oncogenes and lymphokines [Shaw and Kamen, Cell, 46: 659-667 (1986)]. However, the sequence surrounding the first ATG codon in the open reading frame (ORF) did not fit the definition of a translational start site. In addition, as follows from a comparison of the size of the MN clone with that of the corresponding mRNA in a Northern blot (Figure 4), the cDNA was missing about 100 bp from the 5' end of its sequence.

Full-Length cDNA Clone

Attempts to isolate a full-length clone from the original cDNA library failed. Therefore, we performed a rapid amplification of cDNA ends (RACE) using MN-specific primers, R1 and R2, derived from the 5' region of the original cDNA clone. The RACE product was inserted into pBluescript, and the entire population of recombinant plasmids was sequenced with an MN-specific primer ODN1. In that way, we obtained a reliable sequence at the very 5' end of the MN cDNA as shown in Figures 1A-1C [SEQ. ID. NO.: 1].

Specifically, RACE was performed using 5' RACE System [GIBCO BRL; Gaithersburg, MD (USA)] as follows. 1 μ g of mRNA (the same as above) was used as a template for the first strand cDNA synthesis which was primed by the MN-specific antisense oligonucleotide, R1 (5'-TGGGGTTCTTGAGGATCTCCAGGAG-3') [SEQ. ID. NO.: 7]. The first strand product was precipitated twice in the presence of ammonium acetate and a homopolymeric C tail was attached to its 3' end by TdT. Tailed cDNA was then amplified by PCR using a nested primer, R2 (5'-CTCTAACTTCAGGGAGCCCTCTTCTT-3') [SEQ. ID. NO.: 8] and an anchor primer that anneals to the homopolymeric tail (5'-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3') [SEQ. ID. NO.: 9]. Amplified product was digested with BamHI and SalI restriction enzymes and cloned into pBluescript II KS plasmid. After transformation, plasmid DNA was purified from the whole population of transformed cells and used as a template for the sequencing with the MN-specific primer ODN1 [SEQ. ID. NO.: 3; a 29-mer, the sequence for which is shown in Example 10].

Based upon results of the RACE analysis, the full-length MN cDNA sequence was seen to contain a single ORF starting at position 12, with an ATG codon that is in a good context (GCGCATGG) with the rule proposed for translation initiation [Kozak, J. Cell. Biol., 108: 229-241 (1989)].

[See below under Mapping of MN Gene Transcription Initiation Site for fine mapping of the 5' end of the MN gene.] The AT rich 3' untranslated region contains a polyadenylation signal (AATAAA) preceding the end of the cDNA by 10 bp.

5 Surprisingly, the sequence from the original clone as well as from four additional clones obtained from the same cDNA library did not reveal any poly(A) tail. Moreover, as indicated above, just downstream of the poly(A) signal we found an ATTTA motif that is thought to contribute to mRNA
10 instability (Shaw and Kamen, supra). This fact raised the possibility that the poly (A) tail is missing due to the specific degradation of the MN mRNA.

Genomic clones

To study MN regulation, MN genomic clones were
15 isolated. One MN genomic clone (Bd3) was isolated from a human cosmid library prepared from fetal brain using both the MN cDNA probe and the MN-specific primers derived from the 5' end of the cDNA [SEQ. ID. NOS.: 3 and 4; ODN1 AND ODN2; see Example 10]. Sequence analysis revealed that that
20 genomic clone covers a region upstream from a MN transcription start site and ending with the BamHI restriction site localized inside the MN cDNA. Other MN genomic clones can be similarly isolated.

In order to identify the complete genomic region of MN, the human genomic library in Lambda FIX II vector (Stratagene) was prepared from HeLa chromosomal DNA and screened by plaque hybridization using the MN cDNA as
5 described below. Several independent MN recombinant phages were identified, isolated and characterized by restriction mapping and hybridization analyses. Four overlapping recombinants covering the whole genomic region of MN were selected, digested and subcloned into pBluescript. The
10 subclones were then subjected to bidirectional nested deletions and sequencing. DNA sequences were compiled and analyzed by computer using the DNASIS software package.

The details of isolating genomic clones covering the complete genomic region for MN are provided below.
15 Figure 27 provides a schematic of the alignment of MN genomic clones according to the transcription initiation site. Plasmids containing the A4a clone and the XE1 and XE3 subclones were deposited at the American Type Culture Collection (ATCC) at 10801 University Blvd., Manassas,
20 Virginia 20110-2209 (USA) on June 6, 1995, respectively under ATCC Deposit Nos. 97199, 97200, and 97198.

Isolation of Genomic DNA Clones

The Sau3AI human HeLa genomic library was prepared in Lambda FIX II vector [Stratagene; La Jolla, CA (USA)] according to manufacturer's protocol. Human fetal brain cosmid library in SuperCos cosmid was from Stratagene.

5 Recombinant phages or bacteria were plated at 1×10^5 plaque forming units on 22x22 cm Nunc plates or 5×10^4 cells on 150 mm Petri dishes, and plaques or colonies were transferred to Hybond N membranes (Amersham). Hybridization

10 was carried out with the full-length MN cDNA labeled with $[P^{32}]$ PdCTP by the Multiprime DNA labeling method (Amersham) at 65°C in 6 x SSC, 0.5% SDS, 10 x Denhardt's and 0.2 mg/1 ml salmon sperm DNA. Filters were washed twice in 2 x SSC, 0.1% SDS at 65°C for 20 min. The dried filters were exposed

15 to X-ray films, and positive clones were picked up. Phages and bacteria were isolated by 3-4 sequential rounds of screening.

Subcloning and DNA Sequencing

Genomic DNA fragments were subcloned into a

20 pBluescript KS and templates for sequencing were generated by serial nested deletions using Erase-a-Base system (Promega). Sequencing was performed by the dideoxynucleotide chain termination method using T7

sequencing kit (Pharmacia). Nucleotide sequence alignments and analyses were carried out using the DNASIS software package (Hitachi Software Engineering).

Exon-Intron Structure of Complete MN Genomic Region

5 The complete sequence of the overlapping clones contains 10,898 bp (SEQ. ID. NO.: 5). Figure 24 depicts the organization of the human MN gene, showing the location of all 11 exons as well as the 2 upstream and 6 intronic Alu repeat elements. All the exons are small, ranging from 27
10 to 191 bp, with the exception of the first exon which is 445 bp. The intron sizes range from 89 to 1400 bp.

 Table 1 below lists the splice donor and acceptor sequences that conform to consensus splice sequences including the AG-GT motif [Mount, "A catalogue of splice
15 junction sequences," Nucleic Acids Res. 10: 459-472 (1982)].

TABLE 1
Exon-Intron Structure of the Human MN Gene

| | Exon | Size | Genomic Position** | SEQ ID NO | 5'splice donor | SEQ ID No |
|----|--------|------|-----------------------|-----------------|----------------------|-----------------|
| | | | | | | |
| 5 | 1 | 445 | *3507-3951 | 28 | AGAAG gtaagt | 67 |
| | 2 | 30 | 5126-5155 | 29 | TGGAG gtgaga | 68 |
| | 3 | 171 | 5349-5519 | 30 | CAGTC gtgagg | 69 |
| | 4 | 143 | 5651-5793 | 31 | CCGAG gtgagc | 70 |
| | 5 | 93 | 5883-5975 | 32 | TGGAG gtacca | 71 |
| 10 | 6 | 67 | 7376-7442 | 33 | GGAAG gtcagt | 72 |
| | 7 | 158 | 8777-8934 | 34 | AGCAG gtgggc | 73 |
| | 8 | 145 | 9447-9591 | 35 | GCCAG gtacag | 74 |
| | 9 | 27 | 9706-9732 | 36 | TGCTG gtgagt | 75 |
| | 10 | 82 | 10350-10431 | 37 | CACAG gtatta | 76 |
| 15 | 11 | 191 | 10562-10752 | 38 | ATAAT end | |
| | Intron | Size | Genomic Position** | SEQ ID NO | 3'splice acceptor | SEQ ID NO |
| | | | | | | |
| 20 | 1 | 1174 | 3952-5125 | 39 | atacag GGGAT | 77 |
| | 2 | 193 | 5156-5348 | 40 | ccccag GCGAC | 78 |
| | 3 | 131 | 5520-5650 | 41 | acgcag TGCAA | 79 |
| | 4 | 89 | 5794-5882 | 42 | tttcag ATCCA | 80 |
| | 5 | 1400 | 5976-7375 | 43 | ccccag GAGGG | 81 |
| | 6 | 1334 | 7443-8776 | 44 | tcacag GCTCA | 82 |
| | 7 | 512 | 8935-9446 | 45 | ccctag CTCCA | 83 |
| | 8 | 114 | 9592-9705 | 46 | ctccag TCCAG | 84 |
| | 9 | 617 | 9733-10349 | 47 | tcgcag GTGACA | 85 |
| | 10 | 130 | 10432-10561 | 48 | acacag AAGGG | 86 |

** positions are related to nt numbering in whole genomic sequence including the 5' flanking region [Figures 15A-15F]

30 * number corresponds to transcription initiation site determined below by RNase protection assay

A search for sequences related to MN gene in the EMBL Data Library did not reveal any specific homology except for 6 complete and 2 partial Alu-type repeats with homology to Alu sequences ranging from 69.8% to 91% [Jurka
5 and Milosavljevic, "Reconstruction and analysis of human Alu genes," J. Mol. Evol. 32: 105-121 (1991)]. Below under the Characterization of the 5' Flanking Region, also a 222 bp sequence proximal to the 5' end of the genomic region is shown to be closely homologous to a region of the HERV-K
10 LTR.

Mapping of MN Gene Transcription Initiation Site

In our earlier attempt to localize the site of transcription initiation of the MN gene by RACE (above), we obtained a major PCR fragment whose sequence placed the
15 start site 12 bp upstream from the first codon of the ORF. That result was obtained probably due to a preferential amplification of the shortest form of mRNA. Therefore, we used an RNase protection assay (RNP) for fine mapping of the
20 5' end of the MN gene. The probe was a uniformly labeled 470 nucleotide copy RNA (nt -205 to +265) [SEQ. ID. NO.: 55], which was hybridized to total RNA from MN-expressing HeLa and CGL3 cells and analyzed on a sequencing gel. That analysis has shown that the MN gene transcription initiates

at multiple sites, the 5' end of the longest MN transcript being 30 nt longer than that previously characterized by RACE (Figure 18a).

RNase Protection Assay

5 ³²P-labeled RNA probes were prepared with an RNA Transcription kit (Stratagene). In vitro transcription reactions were carried out using 1 µg of the linearized plasmid as a template, 50 µCi of [P³²P]rUTP (800 Ci/mmol), 10 U of either T3 or T7 RNA polymerase and other components
10 of the Transcription Kit following instructions of the supplier. For mapping of the 5' end of MN mRNA, 470 bp NcoI-BamHI fragment (NcoI filled in by Klenow enzyme) of Bd3 clone (nt -205 to +265 related to transcription start) was subcloned to EcoRV-BamHI sites of pBluescript SK+,
15 linearized with HindIII and labeled with T3 RNA polymerase. For the 3' end mRNA analysis, probe, that was prepared using T7 RNA polymerase on KS-dXE3-16 template (one of the nested deletion clones of MN genomic region XE3 subclone) digested with Sau3AI (which cuts exon 11 at position 10,629), was
20 used. Approximately 3 x 10⁵ cpm of RNA probe were used per one RNase protection assay reaction.

RNase protection assays (RNP) were performed using Lysate RNase Protection Kit (USB/Amersham) according to

protocols of the supplier. Briefly, cells were lysed using
Lysis Solution at concentration of approximately 10^7
cells/ml, and 45 μ l of the cell homogenate were used in
RNA/RNA hybridization reactions with 32 P-labeled RNA probes
5 prepared as described above. Following overnight
hybridizations at 42°C, homogenates were treated for 30 min
at 37°C with RNase cocktail mix. Protected RNA duplexes
were run on polyacrylamide/urea denaturing sequencing gels.
Fixed and dried gels were exposed to X-ray film for 24 - 72
10 hours.

Mapping of MN Gene Transcription Termination Site

An RNase protection assay, as described above, was
also used to verify the 3' end of the MN cDNA. That was
important with respect to our previous finding that the cDNA
15 contains a poly(A) signal but lacks a poly(A) tail, which
could be lost during the proposed degradation of MN mRNA due
to the presence of an instability motif in its 3'
untranslated region. RNP analysis of MN mRNA with the
fragment of the genomic clone XE3 covering the region of
20 interest corroborated our data from MN cDNA sequencing,
since the 3' end of the protected fragment corresponded to
the last base of MN cDNA (position 10,752 of the genomic
sequence). That site also meets the requirement for the

presence of a second signal in the genomic sequence that is needed for transcription termination and polyadenylation [McLauchlan et al., Nucleic Acids Res., 13: 1347 (1985)]. Motif TGTGTTAGT (nt 10,759-10,767) corresponds well to both
5 the consensus sequence and the position of that signal within 22 bp downstream from the polyA signal (nt 10,737-10,742).

Characterization of the 5' Flanking Region

The Bd3 genomic clone isolated from human fetal
10 brain cosmid library was found to cover a region of 3.5 kb upstream from the transcription start site of the MN gene. It contains no significant coding region. Two Alu repeats are situated at positions -2587 to -2296 [SEQ. ID NO.: 59] and -1138 to -877 [SEQ. ID NO.: 60] (with respect to the
15 transcription start determined by RNP). The sequence proximal to the 5' end is strongly homologous (91.4% identity) to the U3 region of long terminal repeats of human endogenous retroviruses HERV-K [Ono, M., "Molecular cloning and long terminal repeat sequences of human endogenous
20 retrovirus genes related to types A and B retrovirus genes," J. Virol., 58: 937-944 (1986)]. The LTR-like fragment is 222 bp long with an A-rich tail at its 3' end. Most probably, it represents part of SINE (short interspersed

repeated sequence) type nonviral retroposon derived from
HERV-K [Ono et al., "A novel human nonviral retroposon
derived from an endogenous retrovirus," Nucleic Acids Res.,
15: 8725-8373 (1987)]. There are no sequences

5 corresponding to regulatory elements in this fragment, since
the 3' part of U3, and the entire R and U5 regions of LTR
are absent from the Bd3 genomic clone, and the
glucocorticoid responsive element as well as the enhancer
core sequences are beyond its 5' border.

10 However, two keratinocyte-dependent enhancers were
identified in the sequence downstream from the LTR-like
fragment at positions -3010 and -2814. Those elements are
involved in transcriptional regulation of the E6-E7
oncogenes of human papillomaviruses and are thought to
15 account for their tissue specificity [Cripe et al.,
"Transcriptional regulation of the human papillomavirus-16
E6-E7 promoter by a keratinocyte-dependent enhancer, and by
viral E2 trans-activator and repressor gene products:
implications for cervical carcinogenesis," EMBO J., 6:
20 3745-3753 (1987)].

Nucleotide sequence analysis of the DNA 5' to the
transcription start (from nt -507) revealed no recognizable
TATA box within the expected distance from the beginning of
the first exon (Figure 25). However, the presence of

potential binding sites for transcription factors suggests that this region might contain a promoter for the MN gene. There are several consensus sequences for transcription factors AP1 and AP2 as well as for other regulatory elements, including a p53 binding site [Locker and Buzard, "A dictionary of transcription control sequences," J. DNA Sequencing and Mapping, 1: 3-11 (1990); Imagawa et al., "Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP," Cell, 51: 251-260 (1987); El Deiry et al., "Human genomic DNA sequences define a consensus binding site for p53," Nat. Genet., 1: 44-49 (1992)]. Although the putative promoter region contains 59.3% C+G, it does not have additional attributes of CpG-rich islands that are typical for TATA-less promoters of housekeeping genes [Bird, "CPG-rich islands and the function of DNA methylation," Nature, 321: 209-213 (1986)]. Another class of genes lacking TATA box utilizes the initiator (Inr) element as a promoter. Many of these genes are not constitutively active, but they are rather regulated during differentiation or development. The Inr has a consensus sequence of PyPyPyCAPyPyPyPyPy [SEQ. ID. NO.: 23] and encompasses the transcription start site [Smale and Baltimore, "The 'initiator' as a transcription control element," Cell, 57:

103-113 (1989)]. There are two such consensus sequences in the MN putative promoter; however, they do not overlap the transcription start (Figure 25).

5 In the initial experiments, we were unable to show promoter activity in human carcinoma cells HeLa and CGL3 that express MN, using the 3.5 kb Bd3 fragment and series of its deletion mutants (from nt -933 to -30) [SEQ. ID. NO.: 58] fused to chloramphenicol acetyl transferase (CAT) gene in a transient system. This might indicate that either the
10 promoter activity of the region 5' to the MN transcription start is below the sensitivity of the CAT assay, or additional regulatory elements not present in our constructs are required for driving the expression of MN gene.

15 With respect to this fact, an interesting region was found in the middle of the MN gene. The region is about 1.4 kb in length [nt 4,600-6,000 of the genomic sequence; SEQ. ID. NO.: 49] and spans from the 3' part of the 1st intron to the end of the 5th exon. The region has the character of a typical CpG-rich island, with 62.8% C+G
20 content and 82 CpG: 131 GpC dinucleotides (Figure 26). Moreover, there are multiple putative binding sites for transcription factors AP2 and Sp1 [Locker and Buzard, supra; Briggs et al., "Purification and biochemical characterization of the promoter-specific transcription

factor Sp-1," Science, 234: 47-52 (1986)] concentrated in the center of this area. Particularly the 3rd intron of 131 bp in length contains three Sp1 and three AP2 consensus sequences. That data indicates the possible involvement of
5 that region in the regulation of MN gene expression. However, functionality of that region, as well as other regulatory elements found in the proposed 5' MN promoter, remains to be determined.

MN Promoter Analysis

10 To define sequences necessary for MN gene expression, a series of 5' deletion mutants of the putative promoter region were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. [See Figures 28 and 29.] The pMN-CAT deletion constructs were transfected using a DEAE
15 dextran method for transient expression into HeLa and CGL3 cells. Those cells were used since they naturally express MN protein, and thus, should contain all the required transcription factors.

After 48 hours, crude cell lysates were prepared
20 and the activity of the expressed CAT was evaluated according to acetylation of [¹⁴C]chloramphenicol by thin layer chromatography. However, no MN promoter CAT activity was detected in either the HeLa or the CGL3 cells in a

transient system. On the other hand, reporter CAT plasmids with viral promoters (e.g. pBLV-LTR + tax transactivator, pRSV CAT and pSV2 CAT), that served as positive controls, gave strong signals on the chromatogram. [pSV2 CAT carries
5 the SV40 origin and expresses CAT from the SV40 early promoter (P_E). pRSV CAT expresses CAT from the Rous sarcoma virus (RSV) LTR promoter (P_{LTR}).]

No detectable CAT activity was observed in additional experiments using increasing amounts of
10 transfected plasmids (from 2 to 20 g DNA per dish) and prolonged periods of cell incubation after transcription. Increased cell density also did not improve the results (in contrast to the expectations based on density-dependent expression of native MN protein in HeLa cells). Since we
15 found consensus sequences for transcription factors AP2 and AP1 in the putative MN promoter, we studied the effect of their inducers dexamethasone (1 μ M) and phorbol ester phorbol 12-myristate 13-acetate (PMA 50 ng/ml) on CAT activity. However, the MN promoter was unresponsive to those
20 compounds.

The following provides explanations for the results:

-- the putative MN promoter immediately preceding the transcription initiation site is very weak, and its activity is below the sensitivity of a standard CAT assay;
-- additional sequences (e.g enhancers) are necessary for MN transcription.

To further shed light on the regulation of MN expression at the level of transcription, constructs, analogously prepared to the MN-CAT constructs, are prepared, wherein the MN promoter region is upstream from the neomycin phosphotransferase gene engineered for mammalian expression. Such constructs are then transfected to cells which are subjected to selection with G418. Activity of the promoter is then evaluated on the basis of the number of G418 resistant colonies that result. That method has the capacity to detect activity of a promoter that is 50 to 100 times weaker in comparison to promoters detectable by a CAT assay.

Deduced Amino Acid Sequence

The ORF of the MN cDNA shown in Figures 1A-1C have the coding capacity for a 459 amino acid protein with a calculated molecular weight of 49.7 kd. MN protein has an estimated pI of about 4. As assessed by amino acid sequence analysis, the deduced primary structure of the MN protein

can be divided into four distinct regions. The initial hydrophobic region of 37 amino acids (AA) corresponds to a signal peptide. The mature protein has an N-terminal part of 377 AA, a hydrophobic transmembrane segment of 20 AA and a C-terminal region of 25 AA. Alternatively, the MN protein can be viewed as having five domains as follows: (1) a signal peptide [amino acids (AA) 1-37; SEQ. ID. NO.: 6]; (2) a region of homology to collagen alpha1 chain (AA 38-135; SEQ. ID. NO.: 50); (3) a carbonic anhydrase domain (AA 136-391; SEQ. ID. NO.: 51); (4) a transmembrane region (AA 415-434; SEQ. ID. NO.: 52); and (5) an intracellular C terminus (AA 435-459; SEQ. ID. NO.: 53). (The AA numbers are keyed to Figures 1A-1C.)

More detailed insight into MN protein primary structure disclosed the presence of several consensus sequences. One potential N-glycosylation site was found at position 346 of Figures 1A-1C. That feature, together with a predicted membrane-spanning region are consistent with the results, in which MN was shown to be an N-glycosylated protein localized in the plasma membrane. MN protein sequence deduced from cDNA was also found to contain seven S/TPXX sequence elements [SEQ. ID. NOS.: 25 AND 26] (one of them is in the signal peptide) defined by Suzuki, J. Mol. Biol., 207: 61-84 (1989) as motifs frequently found in gene

regulatory proteins. However, only two of them are composed of the suggested consensus amino acids.

In experiments, the results for which are shown in Figure 21(a), it was determined that MN protein is able to
5 bind zinc cations, as shown by affinity chromatography using Zn-charged chelating sepharose. MN protein immunoprecipitated from HeLa cells by Mab M75 was found to have weak catalytic activity of CA. The CA-like domain of MN has a structural predisposition to serve as a binding
10 site for small soluble domains. Thus, MN protein could mediate some kind of signal transduction.

MN protein from LCMV-infected HeLa cells was shown by using DNA cellulose affinity chromatography [Figure 21(b)] to bind to immobilized double-stranded salmon sperm
15 DNA. The binding activity required both the presence of zinc cations and the absence of a reducing agent in the binding buffer.

Sequence Similarities

Computer analysis of the MN cDNA sequence was
20 carried out using DNASIS and PROSIS (Pharmacia Software packages). GenBank, EMBL, Protein Identification Resource and SWISS-PROT databases were searched for all possible sequence similarities. In addition, a search for proteins

sharing sequence similarities with MN was performed in the MIPS databank with the FastA program [Pearson and Lipman, PNAS (USA), 85: 2444 (1988)].

The MN gene was found to clearly be a novel
5 sequence derived from the human genome. Searches for amino acid sequence similarities in protein databases revealed as the closest homology a level of sequence identity (38.9% in 256 AA or 44% in an 170 AA overlap) between the central part of the MN protein [AAs 136-391 (SEQ. ID. NO: 51)] or 221-
10 390 [SEQ. ID. NO.: 54] of Figures 1A-1C and carbonic anhydrases (CA). However, the overall sequence homology between the cDNA MN sequence and cDNA sequences encoding different CA isoenzymes is in a homology range of 48-50% which is considered by ones in the art to be low.
15 Therefore, the MN cDNA sequence is not closely related to any CA cDNA sequences.

Only very closely related nt sequences having a homology of at least 80-90% would hybridize to each other under stringent conditions. A sequence comparison of the MN
20 cDNA sequence shown in Figures 1A-1C and a corresponding cDNA of the human carbonic anhydrase II (CA II) showed that there are no stretches of identity between the two sequences that would be long enough to allow for a segment of the CA II cDNA sequence having 50 or more nucleotides to hybridize

under stringent hybridization conditions to the MN cDNA or vice versa.

Although MN deduced amino acid sequences show some homology to known carbonic anhydrases, they differ from them in several respects. Seven carbonic anhydrases are known [Dodgson et al. (eds.), The Carbonic Anhydrases, (Plenum Press; New York/London (1991))]. All of the known carbonic anhydrases are proteins of about 30 kd, smaller than the p54/58N-related products of the MN gene. Further, the carbonic anhydrases do not form oligomers as do the MN-related proteins.

The N-terminal part of the MN protein (AA 38-135; SEQ. ID. NO.: 50) shows a 27-30% identity with human collagen alpha1 chain, which is an important component of the extracellular matrix.

MN Twin Protein

The possibility that the 4 kd difference between the molecular weights of the two MN proteins is caused by different glycosylation was ruled out, since after in vitro treatment with endoglycosidases H and F, respectively, both peptide portions lost about 3 kd in weight. This result indicates, in addition, that the molecular weight of the smaller 54 kd MN protein without its 3 kd sugar moiety,

roughly corresponds to the molecular weight of MN calculated from the full-length cDNA. Western blot analysis of MN proteins from cervical carcinoma and normal stomach shows that in both tissues MN protein consists of two 54 and 58 kd peptide portions.

To determine whether both p54/58N proteins were encoded by one gene, antisense ODNs were used to inhibit specifically MN gene expression. [Such use of antisense ODNs is reviewed in Stein and Cohen, Cancer Res., 48: 2659-2668 (1988).] Those experiments are detailed in Example 10. The findings indicated that cultivation of HeLa cells with ODNs resulted in a considerable inhibition of p54/58N synthesis, whereas the amount of different HeLa cell proteins produced remained approximately the same. Further, and importantly, on immunoblotting, the specific inhibition by ODNs affected both of the p54/58N proteins (Figure 3). Thus, it was concluded that the MN gene that was cloned codes for both of the p54/58N proteins in HeLa cells.

MN Proteins and/or Polypeptides

The phrase "MN proteins and/or polypeptides" (MN proteins/polypeptides) is herein defined to mean proteins and/or polypeptides encoded by an MN gene or fragments thereof. An exemplary and preferred MN protein according to

this invention has the deduced amino acid sequence shown in Figures 1A-1C. Preferred MN proteins/polypeptides are those proteins and/or polypeptides that have substantial homology with the MN protein shown in Figures 1A-1C. For example,
5 such substantially homologous MN proteins/polypeptides are those that are reactive with the MN-specific antibodies of this invention, preferably the Mabs M75, MN12, MN9 and MN7 or their equivalents.

A "polypeptide" is a chain of amino acids
10 covalently bound by peptide linkages and is herein considered to be composed of 50 or less amino acids. A "protein" is herein defined to be a polypeptide composed of more than 50 amino acids.

MN proteins exhibit several interesting features:
15 cell membrane localization, cell density dependent expression in HeLa cells, correlation with the tumorigenic phenotype of HeLa x fibroblast somatic cell hybrids, and expression in several human carcinomas among other tissues. As demonstrated herein, for example, in Example 13, MN
20 protein can be found directly in tumor tissue sections but not in general in counterpart normal tissues (exceptions noted infra in Example 13 as in normal stomach tissues). MN is also expressed sometimes in morphologically normal appearing areas of tissue specimens exhibiting dysplasia

and/or malignancy. Taken together, these features suggest a possible involvement of MN in the regulation of cell proliferation, differentiation and/or transformation.

It can be appreciated that a protein or
5 polypeptide produced by a neoplastic cell in vivo could be altered in sequence from that produced by a tumor cell in cell culture or by a transformed cell. Thus, MN proteins and/or polypeptides which have varying amino acid sequences including without limitation, amino acid substitutions,
10 extensions, deletions, truncations and combinations thereof, fall within the scope of this invention. It can also be appreciated that a protein extant within body fluids is subject to degradative processes, such as, proteolytic processes; thus, MN proteins that are significantly
15 truncated and MN polypeptides may be found in body fluids, such as, sera. The phrase "MN antigen" is used herein to encompass MN proteins and/or polypeptides.

It will further be appreciated that the amino acid sequence of MN proteins and polypeptides can be modified by
20 genetic techniques. One or more amino acids can be deleted or substituted. Such amino acid changes may not cause any measurable change in the biological activity of the protein or polypeptide and result in proteins or polypeptides which

are within the scope of this invention, as well as, MN muteins.

The MN proteins and polypeptides of this invention can be prepared in a variety of ways according to this invention, for example, recombinantly, synthetically or
5 otherwise biologically, that is, by cleaving longer proteins and polypeptides enzymatically and/or chemically. A preferred method to prepare MN proteins is by a recombinant means. Particularly preferred methods of recombinantly producing MN proteins are described below for the GEX-3X-MN,
10 MN 20-19, MN-Fc and MN-PA proteins.

Recombinant Production of MN Proteins and Polypeptides

A representative method to prepare the MN proteins shown in Figures 1A-1C or fragments thereof would be to insert the full-length or an appropriate fragment of MN cDNA
15 into an appropriate expression vector as exemplified below. The fusion protein GEX-3X-MN expressed from XL1-Blue cells is nonglycosylated. Representative of a glycosylated, recombinantly produced MN protein is the MN 20-19 protein expressed from insect cells. The MN 20-19 protein was also
20 expressed in a nonglycosylated form in E. coli using the expression plasmid pEt-22b [Novagen].

Baculovirus Expression Systems. Recombinant baculovirus express vectors have been developed for infection into several types of insect cells. For example, recombinant baculoviruses have been developed for among
5 others: Aedes aegypti, Autographa californica, Bombyx mor, Drosophila melanogaster, Heliothis zea, Spodoptera frugiperda, and Trichoplusia ni [PCT Pub. No. WO 89/046699; Wright, Nature, 321: 718 (1986); Fraser et al., In Vitro Cell Dev. Biol., 25: 225 (1989). Methods of introducing
10 exogenous DNA into insect hosts are well-known in the art. DNA transfection and viral infection procedures usually vary with the insect genus to be transformed. See, for example, Autographa [Carstens et al., Virology, 101: 311 (1980)]; Spodoptera [Kang, "Baculovirus Vectors for Expression of
15 Foreign Genes," in: Advances in Virus Research, 35 (1988)]; and Heliothis (virescens) [PCT Pub. No. WO 88/02030].

A wide variety of other host-cloning vector combinations may be usefully employed in cloning the MN DNA isolated as described herein. For example, useful cloning
20 vehicles may include chromosomal, nonchromosomal and synthetic DNA sequences such as various known bacterial plasmids such as pBR322, other E. coli plasmids and their derivatives and wider host range plasmids such as RP4, phage DNA, such as, the numerous derivatives of phage lambda,

e.g., NB989 and vectors derived from combinations of plasmids and phage DNAs such as plasmids which have been modified to employ phage DNA expression control sequences.

Useful hosts may be eukaryotic or prokaryotic and
5 include bacterial hosts such as E. coli and other bacterial strains, yeasts and other fungi, animal or plant hosts such as animal or plant cells in culture, insect cells and other hosts. Of course, not all hosts may be equally efficient. The particular selection of host-cloning vehicle combination
10 may be made by those of skill in the art after due consideration of the principles set forth herein without departing from the scope of this invention.

The particular site chosen for insertion of the selected DNA fragment into the cloning vehicle to form a
15 recombinant DNA molecule is determined by a variety of factors. These include size and structure of the protein or polypeptide to be expressed, susceptibility of the desired protein or polypeptide to endoenzymatic degradation by the host cell components and contamination by its proteins,
20 expression characteristics such as the location of start and stop codons, and other factors recognized by those of skill in the art.

The recombinant nucleic acid molecule containing the MN gene, fragment thereof, or cDNA therefrom, may be

employed to transform a host so as to permit that host (transformant) to express the structural gene or fragment thereof and to produce the protein or polypeptide for which the hybrid DNA encodes. The recombinant nucleic acid molecule may also be employed to transform a host so as to permit that host on replication to produce additional recombinant nucleic acid molecules as a source of MN nucleic acid and fragments thereof. The selection of an appropriate host for either of those uses is controlled by a number of factors recognized in the art. These include, for example, compatibility with the chosen vector, toxicity of the co-products, ease of recovery of the desired protein or polypeptide, expression characteristics, biosafety and costs.

Where the host cell is a procaryote such as E. coli, competent cells which are capable of DNA uptake are prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl_2 method by well known procedures. Transformation can also be performed after forming a protoplast of the host cell.

Where the host used is an eukaryote, transfection methods such as the use of a calcium phosphate-precipitate, electroporation, conventional mechanical procedures such as microinjection, insertion of a plasmid encapsulated in red

blood cell ghosts or in liposomes, treatment of cells with agents such as lysophosphatidyl-choline or use of virus vectors, or the like may be used.

The level of production of a protein or
5 polypeptide is governed by three major factors: (1) the number of copies of the gene or DNA sequence encoding for it within the cell; (2) the efficiency with which those gene and sequence copies are transcribed and translated; and (3) the stability of the mRNA. Efficiencies of transcription
10 and translation (which together comprise expression) are in turn dependent upon nucleotide sequences, normally situated ahead of the desired coding sequence. Those nucleotide sequences or expression control sequences define, inter alia, the location at which an RNA polymerase interacts to
15 initiate transcription (the promoter sequence) and at which ribosomes bind and interact with the mRNA (the product of transcription) to initiate translation. Not all such expression control sequences function with equal efficiency. It is thus of advantage to separate the specific coding
20 sequences for the desired protein from their adjacent nucleotide sequences and fuse them instead to known expression control sequences so as to favor higher levels of expression. This having been achieved, the newly engineered DNA fragment may be inserted into a multicopy plasmid or a

bacteriophage derivative in order to increase the number of gene or sequence copies within the cell and thereby further improve the yield of expressed protein.

Several expression control sequences may be
5 employed. These include the operator, promoter and ribosome binding and interaction sequences (including sequences such as the Shine-Dalgarno sequences) of the lactose operon of E. coli ("the lac system"), the corresponding sequences of the tryptophan synthetase system of E. coli ("the trp system"),
10 a fusion of the trp and lac promoter ("the tac system"), the major operator and promoter regions of phage lambda ($O_L P_L$ and $O_R P_R$), and the control region of the phage fd coat protein. DNA fragments containing these sequences are excised by cleavage with restriction enzymes from the DNA
15 isolated from transducing phages that carry the lac or trp operons, or from the DNA of phage lambda or fd. Those fragments are then manipulated in order to obtain a limited population of molecules such that the essential controlling sequences can be joined very close to, or in juxtaposition
20 with, the initiation codon of the coding sequence.

The fusion product is then inserted into a cloning vehicle for transformation or transfection of the appropriate hosts and the level of antigen production is measured. Cells giving the most efficient expression may be

thus selected. Alternatively, cloning vehicles carrying the lac, trp or lambda P_L control system attached to an initiation codon may be employed and fused to a fragment containing a sequence coding for a MN protein or polypeptide such that the gene or sequence is correctly translated from the initiation codon of the cloning vehicle.

The phrase "recombinant nucleic acid molecule" is herein defined to mean a hybrid nucleotide sequence comprising at least two nucleotide sequences, the first sequence not normally being found together in nature with the second.

The phrase "expression control sequence" is herein defined to mean a sequence of nucleotides that controls and regulates expression of structural genes when operatively linked to those genes.

The following are representative examples of genetically engineering MN proteins of this invention. The descriptions are exemplary and not meant to limit the invention in any way.

20 Production of Fusion Protein GEX-3X-MN

To confirm whether the partial cDNA clone codes for the p54/58N-specific protein, it was subcloned into the bacterial expression vector pGEX-3X [Pharmacia; Upsala,

Sweden], constructed to express a fusion protein containing the C-terminus of glutathione S-transferase. The partial cDNA insert from the above-described pBluescript-MN was released by digesting the plasmid DNA by NotI. It was then
5 treated with S1 nuclease to obtain blunt ends and then cloned into a dephosphorylated SmaI site of pGEX-3X [Pharmacia]. After transformation of XL1-Blue cells [E. coli strain; Stratagene] and induction with IPTG, a fusion protein was obtained.

10 The fusion protein--MN glutathione S-transferase (GEX-3X-MN) was purified by affinity chromatography on Glutathione-S-Sepharose 4B [Pharmacia]. Twenty micrograms of the purified recombinant protein in each of two parallel samples were separated by SDS-PAGE on a 10% gel. One of the
15 samples (A) was stained with Coomassie brilliant blue, whereas the other (B) was blotted onto a Hybond C membrane [Amersham]. The blot was developed by autoradiography with ¹²⁵I-labeled MAb M75. The results are shown in Figure 2.

 SDS-PAGE analysis provided an interesting result:
20 a number of protein bands with different molecular weights (Figure 2A). A similar SDS-PAGE pattern was obtained with another representative fusion protein produced according to this invention, beta-galactosidase-MN that was expressed from lambda gt11 lysogens.

By immunoblotting, a similar pattern was obtained:
all the bands seen on stained SDS-PAGE gel reacted with the
MN-specific MAb M75 (Figure 2B), indicating that all the
protein bands are MN-specific. Also, that result indicates
5 that the binding site for MAb M75 is on the N-terminal part
of the MN protein, which is not affected by frameshifts.

As shown in Example 8 below, the fusion protein
GEX-3X-MN was used in radioimmunoassays for MN-specific
antibodies and for MN antigen.

10 Expression of MN 20-19 Protein

Another representative, recombinantly produced MN
protein of this invention is the MN 20-19 protein which,
when produced in baculovirus-infected Sf9 cells [Spodoptera
frugiperda cells; Clontech; Palo Alto, CA (USA)], is
15 glycosylated. The MN 20-19 protein misses the putative
signal peptide (AAs 1-37) of SEQ. ID. NO.: 6 (Figures 1A-
1C), has a methionine (Met) at the N-terminus for
expression, and a Leu-Glu-His-His-His-His-His [SEQ. ID
NO.: 22] added to the C-terminus for purification. In
20 order to insert the portion of the MN coding sequence for
the GEX-3X-MN fusion protein into alternate expression
systems, a set of primers for PCR was designed. The primers
were constructed to provide restriction sites at each end of

the coding sequence, as well as in-frame start and stop codons. The sequences of the primers, indicating restriction enzyme cleavage sites and expression landmarks, are shown below.

5 Primer #20:N-terminus

 └Translation start
5'GTCGCTAGCTCCATGGGTCATATGCAGAGGTTGCCCCGATGCAG 3'
 NheI NcoI NdeI -MN cDNA #1 [SEQ. ID. NO. 17]

Primer #19:C-terminus

10 └Translation stop
5'GAAGATCTCTTACTCGAGCATTTCTCCAAGATCCAGCCTCTAGG 3'
 BglII XhoI -MN cDNA [SEQ. ID. NO. 18]

The SEQ. ID. NOS.: 17 and 18 primers were used to amplify the MN coding sequence present in the pGEX-3X-MN vector using standard PCR techniques. The resulting PCR product (termed MN 20-19) was electrophoresed on a 0.5% agarose/1X TBE gel; the 1.3 kb band was excised; and the DNA recovered using the Gene Clean II kit according to the manufacturer's instructions [Bio101; LaJolla, CA (USA)].

20 MN 20-19 and plasmid pET-22b [Novagen, Inc.; Madison, WI (USA)] were cleaved with the restriction enzymes NdeI and XhoI, phenol-chloroform extracted, and the

appropriate bands recovered by agarose gel electrophoresis as above. The isolated fragments were ethanol co-precipitated at a vector:insert ratio of 1:4. After resuspension, the fragments were ligated using T4 DNA
5 ligase. The resulting product was used to transform competent Novablue E. coli cells [Novagen, Inc.]. Plasmid mini-preps [Magic Minipreps; Promega] from the resultant ampicillin resistant colonies were screened for the presence of the correct insert by restriction mapping. Insertion of
10 the gene fragment into the pET-22b plasmid using the NdeI and XhoI sites added a 6-histidine tail to the protein that could be used for affinity isolation.

To prepare MN 20-19 for insertion into the baculovirus expression system, the MN 20-19 gene fragment
15 was excised from pET-22b using the restriction endonucleases XbaI and PvuI. The baculovirus shuttle vector pBacPAK8 [Clontech] was cleaved with XbaI and PacI. The desired fragments (1.3 kb for MN 20-19 and 5.5 kb for pBacPAK8) were isolated by agarose gel electrophoresis, recovered using
20 Gene Clean II, and co-precipitated at an insert:vector ratio of 2.4:1.

After ligation with T4 DNA ligase, the DNA was used to transform competent NM522 E. coli cells (Stratagene). Plasmid mini-preps from resultant ampicillin

resistant colonies were screened for the presence of the correct insert by restriction mapping. Plasmid DNA from an appropriate colony and linearized BacPAK6 baculovirus DNA [Clontech] were used to transform Sf9 cells by standard techniques. Recombination produced BacPAK viruses carrying the MN 20-19 sequence. Those viruses were plated onto Sf9 cells and overlaid with agar.

Plaques were picked and plated onto Sf9 cells. The conditioned media and cells were collected. A small aliquot of the conditioned media was set aside for testing. The cells were extracted with PBS with 1% Triton X100.

The conditioned media and the cell extracts were dot blotted onto nitrocellulose paper. The blot was blocked with 5% non-fat dried milk in PBS. Mab M75 were used to detect the MN 20-19 protein in the dot blots. A rabbit anti-mouse Ig-HRP was used to detect bound Mab M75. The blots were developed with TMB/H₂O₂ with a membrane enhancer [KPL; Gaithersburg, MD (USA)]. Two clones producing the strongest reaction on the dot blots were selected for expansion. One was used to produce MN 20-19 protein in High Five cells [Invitrogen Corp., San Diego, CA (USA); BTI-TN-5BI-4; derived from Trichoplusia ni egg cell homogenate]. MN 20-19 protein was purified from the conditioned media from the virus infected High Five cells.

The MN 20-19 protein was purified from the conditioned media by immunoaffinity chromatography. 6.5 mg of Mab M75 was coupled to 1 g of Tresyl activated Toyopearl™ [Tosoh, Japan (#14471)] (solid support in bead form). Approximately 150 ml of the conditioned media was run through the M75-Toyopearl™ (solid support in bead form) column. The column was washed with PBS, and the MN 20-19 protein was eluted with 1.5 M MgCl. The eluted protein was then dialyzed against PBS.

10 Fusion Proteins with C-Terminal Part Including Transmembrane Region Replaced by Fc or PA

MN fusion proteins in which the C terminal part including the transmembrane region is replaced by the Fc fragment of human IgG or by Protein A were constructed. Such fusion proteins are useful to identify MN binding protein(s). In such MN chimaeras, the whole N-terminal part of MN is accessible to interaction with heterologous proteins, and the C terminal tag serves for simple detection and purification of protein complexes.

20 Fusion Protein MN-PA (Protein A)

In a first step, the 3' end of the MN cDNA encoding the transmembrane region of the MN protein was

deleted. The plasmid pFLMN (e.g. pBluescript with full length MN cDNA) was cleaved by *EcoRI* and blunt ended by *S1* nuclease. Subsequent cleavage by *SacI* resulted in the removal of the *EcoRI-SacI* fragment. The deleted fragment
5 was then replaced by a Protein A coding sequence that was derived from plasmid pEZZ (purchased from Pharmacia), which had been cleaved with *RsaI* and *SacI*. The obtained MN-PA construct was subcloned into a eukaryotic expression vector pSG5C (described in Example 15), and was then ready for
10 transfection experiments.

Fusion Protein MN-Fc

The cloning of the fusion protein MN-Fc was rather complicated due to the use of a genomic clone containing the Fc fragment of human IgG which had a complex structure in
15 that it contained an enhancer, a promoter, exons and introns. Moreover, the complete sequence of the clone was not available. Thus, it was necessary to ensure the correct in-phase splicing and fusion of MN to the Fc fragment by the addition of a synthetic splice donor site (SSDS) designed
20 according to the splicing sequences of the MN gene.

The construction procedure was as follows:

1. Plasmid pMH4 (e.g. pSV2gpt containing a genomic clone of the human IgG Fc region) was cleaved by

*Bam*HI in order to get a 13 kb fragment encoding Fc. [In pSV2*gpt*, the *E. coli* xanthine-guanine phosphoribosyl transferase gene (*gpt*) is expressed using the SV40 early promoter (P_E) located in the SV40 origin, the SV40 small T intron, and the SV40 polyadenylation site.]

2. At the same time, plasmid pFLMN (with full length MN cDNA) was cleaved by *Sal*I-*Eco*RI. The released fragment was purified and ligated with a synthetic adapter *Eco*RI-*Bgl*III containing a synthetic splice donor site (SSDS).

10 3. Simultaneously, the plasmid pBKCMV was cleaved by *Sal*I-*Bam*HI. Then advantage was taken of the fact that the *Bam*HI cohesive ends (of the Fc coding fragment) are compatible with the *Bgl*III ends of the SSDS, and Fc was ligated to MN. The MN-Fc ligation product was then inserted
15 into pBKCMV by directional cloning through the *Sal*I and *Bam*HI sites.

Verification of the correct orientation and in-phase fusion of the obtained MN-Fc chimaeric clones was problematic in that the sequence of Fc was not known. Thus,
20 functional constructs are selected on the basis of results of transient eukaryotic expression analyses.

Synthetic and Biologic Production of
MN Proteins and Polypeptides

MN proteins and polypeptides of this invention may be prepared not only by recombinant means but also by
5 synthetic and by other biologic means. Synthetic formation of the polypeptide or protein requires chemically synthesizing the desired chain of amino acids by methods well known in the art. Exemplary of other biologic means to prepare the desired polypeptide or protein is to subject to
10 selective proteolysis a longer MN polypeptide or protein containing the desired amino acid sequence; for example, the longer polypeptide or protein can be split with chemical reagents or with enzymes.

Chemical synthesis of a peptide is conventional in
15 the art and can be accomplished, for example, by the Merrifield solid phase synthesis technique [Merrifield, J., Am. Chem. Soc., 85: 2149-2154 (1963); Kent et al., Synthetic Peptides in Biology and Medicine, 29 f.f. eds. Alitalo et al., (Elsevier Science Publishers 1985); and
20 Haug, J.D., "Peptide Synthesis and Protecting Group Strategy", American Biotechnology Laboratory, 5(1): 40-47 (Jan/Feb. 1987)].

Techniques of chemical peptide synthesis include using automatic peptide synthesizers employing commercially

available protected amino acids, for example, Biosearch [San
Rafael, CA (USA)] Models 9500 and 9600; Applied Biosystems,
Inc. [Foster City, CA (USA)] Model 430; Milligen [a division
of Millipore Corp.; Bedford, MA (USA)] Model 9050; and Du
5 Pont's RAMP (Rapid Automated Multiple Peptide Synthesis) [Du
Pont Compass, Wilmington, DE (USA)].

Regulation of MN Expression and MN Promoter

MN appears to be a novel regulatory protein that
is directly involved in the control of cell proliferation
10 and in cellular transformation. In HeLa cells, the
expression of MN is positively regulated by cell density.
Its level is increased by persistent infection with LCMV.
In hybrid cells between HeLa and normal fibroblasts, MN
expression correlates with tumorigenicity. The fact that MN
15 is not present in nontumorigenic hybrid cells (CGL1), but is
expressed in a tumorigenic segregant lacking chromosome 11,
indicates that MN is negatively regulated by a putative
suppressor in chromosome 11.

Evidence supporting the regulatory role of MN
20 protein was found in the generation of stable transfectants
of NIH 3T3 cells that constitutively express MN protein as
described in Example 15. As a consequence of MN expression,
the NIH 3T3 cells acquired features associated with a

transformed phenotype: altered morphology, increased saturation density, proliferative advantage in serum-reduced media, enhanced DNA synthesis and capacity for anchorage-independent growth. Further, as shown in Example 16, flow
5 cytometric analyses of asynchronous cell populations indicated that the expression of MN protein leads to accelerated progression of cells through G1 phase, reduction of cell size and the loss of capacity for growth arrest under inappropriate conditions. Also, Example 16 shows that
10 MN expressing cells display a decreased sensitivity to the DNA damaging drug mitomycin C.

Nontumorigenic human cells, CGL1 cells, were also transfected with the full-length MN cDNA. The same pSG5C-MN construct in combination with pSV2neo plasmid as used to
15 transfect the NIH 3T3 cells (Example 15) was used. Also the protocol was the same except that the G418 concentration was increased to 1000 $\mu\text{g/ml}$.

Out of 15 MN-positive clones (tested by SP-RIA and Western blotting), 3 were chosen for further analysis. Two
20 MN-negative clones isolated from CGL1 cells transfected with empty plasmid were added as controls. Initial analysis indicates that the morphology and growth habits of MN-transfected CGL1 cells are not changed dramatically, but

their proliferation rate and plating efficiency is increased.

MN cDNA and promoter. When the promoter region from the MN genomic clone, isolated as described above, was linked to MN cDNA and transfected into CGL1 hybrid cells, expression of MN protein was detectable immediately after selection. However, then it gradually ceased, indicating thus an action of a feedback regulator. The putative regulatory element appeared to be acting via the MN promoter, because when the full-length cDNA (not containing the promoter) was used for transfection, no similar effect was observed.

An "antisense" MN cDNA/MN promoter construct was used to transfect CGL3 cells. The effect was the opposite of that of the CGL1 cells transfected with the "sense" construct. Whereas the transfected CGL1 cells formed colonies several times larger than the control CGL1, the transfected CGL3 cells formed colonies much smaller than the control CGL3 cells.

For those experiments, the part of the promoter region that was linked to the MN cDNA through a BamHI site was derived from a NcoI - BamHI fragment of the MN genomic clone [Bd3] and represents a region a few-hundred bp upstream from the transcription initiation site. After the

ligation, the joint DNA was inserted into a pBK-CMV expression vector [Stratagene]. The required orientation of the inserted sequence was ensured by directional cloning and subsequently verified by restriction analysis. The

5 tranfection procedure was the same as used in transfecting the NIH 3T3 cells (Example 15), but co-transfection with the pSV2neo plasmid was not necessary since the neo selection marker was already included in the pBK-CMV vector.

After two weeks of selection in a medium

10 containing G418, remarkable differences between the numbers and sizes of the colonies grown were evident as noted above. Immediately following the selection and cloning, the MN-transfected CGL1 and CGL3 cells were tested by SP-RIA for expression and repression of MN, respectively. The isolated

15 transfected CGL1 clones were MN positive (although the level was lower than obtained with the full-length cDNA), whereas MN protein was almost absent from the transfected CGL3 clones. However, in subsequent passages, the expression of MN in transfected CGL1 cells started to cease, and was then

20 blocked perhaps evidencing a control feedback mechanism.

As a result of the very much lowered proliferation of the transfected CGL3 cells, it was difficult to expand the majority of cloned cells (according to SP-RIA, those with the lowest levels of MN), and they were lost during

passaging. However, some clones overcame that problem and again expressed MN. It is possible that once those cells reached a higher quantity, that the level of endogenously produced MN mRNA increased over the amount of ectopically expressed antisense mRNA.

Transformation and Reversion

As illustrated in Examples 15 and 16, vertebrate cells transfected with MN cDNA in suitable vectors show striking morphologic transformation. Transformed cells may be very small, densely packed, slowly growing, with basophilic cytoplasm and enlarged Golgi apparatus. However, it has been found that transformed clones revert over time, for example, within 3-4 weeks, to nearly normal morphology, even though the cells may be producing MN protein at high levels. MN protein is biologically active even in yeast cells; depending upon the level of its expression, it stimulates or retards their growth and induces morphologic alterations.

Full-length MN cDNA was inserted into pGD, a MLV-derived vector, which together with standard competent MLV (murine leukemia virus), forms an infectious, transmissible complex [pGD-MN + MLV]. That complex also transforms vertebrate cells, such as, NIH 3T3 cells and mouse embryo

fibroblasts BALB/c, which also revert to nearly normal morphology. Such revertants again contain MN protein and produce the [pGD-MN + MLV] artificial virus complex, which retains its transforming capacity. Thus, reversion of MN-
5 transformed cells is apparently not due to a loss, silencing or mutation of MN cDNA, but may be the result of the activation of suppressor gene(s).

Nucleic Acid Probes and Test Kits

Nucleic acid probes of this invention are those
10 comprising sequences that are complementary or substantially complementary to the MN cDNA sequence shown in Figures 1A-1C or to other MN gene sequences, such as, the complete genomic sequence of Figures 15A-15F [SEQ. ID. NO.: 5] and the putative promoter sequence [SEQ. ID. NO.: 27 of Figure 25].
15 The phrase "substantially complementary" is defined herein to have the meaning as it is well understood in the art and, thus, used in the context of standard hybridization conditions. The stringency of hybridization conditions can be adjusted to control the precision of complementarity.
20 Exemplary are the stringent hybridization conditions used in Examples 11 and 12. Two nucleic acids are, for example, substantially complementary to each other, if they hybridize to each other under such stringent hybridization conditions.

Stringent hybridization conditions are considered herein to conform to standard hybridization conditions understood in the art to be stringent. For example, it is generally understood that stringent conditions encompass
5 relatively low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of 50°C to 70°C. Less stringent conditions, such as, 0.15 M to 0.9 M salt at temperatures ranging from 20°C to 55°C can be made more stringent by adding increasing amounts of formamide,
10 which serves to destabilize hybrid duplexes as does increased temperature.

Exemplary stringent hybridization conditions are described in Examples 11 and 12, infra; the hybridizations therein were carried out "in the presence of 50% formamide
15 at 42°C." [See Sambrook et al., Molecular Cloning: A Laboratory Manual, pages 1.91 and 9.47-9.51 (Second Edition, Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY; 1989); Maniatis et al., Molecular Cloning: A Laboratory Manual, pages 387-389 (Cold Spring Harbor Laboratory; Cold
20 Spring Harbor, NY; 1982); Tsuchiya et al., Oral Surgery, Oral Medicine, Oral Pathology, 71(6): 721-725 (June 1991).]

Preferred nucleic acid probes of this invention are fragments of the isolated nucleic acid sequences that encode MN proteins or polypeptides according to this

invention. Preferably those probes are composed of at least fifty nucleotides.

However, nucleic acid probes of this invention need not hybridize to a coding region of MN. For example,
5 nucleic acid probes of this invention may hybridize partially or wholly to a non-coding region of the genomic sequence shown in Figures 15A-15F [SEQ. ID. NO.: 5]. Conventional technology can be used to determine whether fragments of SEQ. ID. NO.: 5 or related nucleic acids are
10 useful to identify MN nucleic acid sequences. [See, for example, Benton and Davis, supra and Fuscoe et al., supra.]

Areas of homology of the MN nt sequence to other non-MN nt sequences are indicated above. In general, nucleotide sequences that are not in the Alu or LTR-like
15 regions of preferably 29 bases or more, or still more preferably of 50 bases or more, can be routinely tested and screened and found to hybridize under stringent conditions to only MN nucleotide sequences. Further, not all homologies within the Alu-like MN genomic sequences are so
20 close to Alu repeats as to give a hybridization signal under stringent hybridization conditions. The percent of homology between MN Alu-like regions and a standard Alu-J sequence are indicated as follows:

| | <u>Region of Homology within</u> <u>MN Genomic Sequence</u> <u>[SEQ. ID. NO.: 5;</u> <u>Figures 15A-15F]</u> | <u>SEQ.</u> <u>ID.</u> <u>NOS.</u> | <u>% Homology to</u> <u>Entire Alu-J</u> <u>Sequence</u> |
|----|---|--|---|
| 5 | 921-1212 | 59 | 89.1% |
| | 2370-2631 | 60 | 78.6% |
| | 4587-4880 | 61 | 90.1% |
| | 6463-6738 | 62 | 85.4% |
| | 7651-7939 | 63 | 91.0% |
| 10 | 9020-9317 | 64 | 69.8% |
| | | | <u>% Homology to</u> <u>One Half of</u> <u>Alu-J Sequence</u> |
| | 8301-8405 | 65 | 88.8% |
| | 10040-10122 | 66 | 73.2%. |

Nucleic acid probes of this invention can be used to detect MN DNA and/or RNA, and thus can be used to test for the presence or absence of MN genes, and amplification(s), mutation(s) or genetic rearrangements of MN genes in the cells of a patient. For example, overexpression of an MN gene may be detected by Northern blotting and RNase protection analysis using probes of this invention. Gene alterations, as amplifications, translocations, inversions, and deletions among others, can be detected by using probes of this invention for in situ hybridization to chromosomes from a patient's cells, whether

in metaphase spreads or interphase nuclei. Southern blotting could also be used with the probes of this invention to detect amplifications or deletions of MN genes. Restriction Fragment Length Polymorphism (RFLP) analysis using said probes is a preferred method of detecting gene alterations, mutations and deletions. Said probes can also be used to identify MN proteins and/or polypeptides as well as homologs or near homologs thereto by their hybridization to various mRNAs transcribed from MN genes in different tissues.

Probes of this invention thus can be useful diagnostically/ prognostically. Said probes can be embodied in test kits, preferably with appropriate means to enable said probes when hybridized to an appropriate MN gene or MN mRNA target to be visualized. Such samples include tissue specimens including smears, body fluids and tissue and cell extracts.

PCR Assays. To detect relatively large genetic rearrangements, hybridization tests can be used. To detect relatively small genetic rearrangements, as, for example, small deletions or amplifications, or point mutations, the polymerase chain reaction (PCR) would preferably be used. [U.S. Patent Nos. 4,800,159; 4,683,195; 4,683,202; and

Chapter 14 of Sambrook et al., Molecular Cloning: A Laboratory Manual, supra

5 An exemplary assay would use cellular DNA from normal and cancerous cells, which DNA would be isolated and amplified employing appropriate PCR primers. The PCR products would be compared, preferably initially, on a sizing gel to detect size changes indicative of certain genetic rearrangements. If no differences in sizes are noted, further comparisons can be made, preferably using, 10 for example, PCR-single-strand conformation polymorphism (PCR-SSCP) assay or a denaturing gradient gel electrophoretic assay. [See, for example, Hayashi, K., "PCR-SSCP: A Simple and Sensitive Method for Detection of Mutations in the Genomic DNA," in PCR Methods and 15 Applications, 1: 34-38 (1991); and Meyers et al., "Detection and Localization of Single Base Changes by Denaturing Gradient Gel Electrophoresis," Methods in Enzymology, 155: 501 (1987).]

Assays

20 Assays according to this invention are provided to detect and/or quantitate MN antigen or MN-specific antibodies in vertebrate samples, preferably mammalian samples, more preferably human samples. Such samples

include tissue specimens, body fluids, tissue extracts and cell extracts. MN antigen may be detected by immunoassay, immunohistochemical staining, immunoelectron and scanning microscopy using immunogold among other techniques.

5 Preferred tissue specimens to assay by immunohistochemical staining include cell smears, histological sections from biopsied tissues or organs, and imprint preparations among other tissue samples. Such tissue specimens can be variously maintained, for example, 10 they can be fresh, frozen, or formalin-, alcohol- or acetone- or otherwise fixed and/or paraffin-embedded and deparaffinized. Biopsied tissue samples can be, for example, those samples removed by aspiration, bite, brush, cone, chorionic villus, endoscopic, excisional, incisional, 15 needle, percutaneous punch, and surface biopsies, among other biopsy techniques.

 Preferred cervical tissue specimens include cervical smears, conization specimens, histologic sections from hysterectomy specimens or other biopsied cervical 20 tissue samples. Preferred means of obtaining cervical smears include routine swab, scraping or cytobrush techniques, among other means. More preferred are cytobrush or swab techniques. Preferably, cell smears are made on

microscope slides, fixed, for example, with 55% EtOH or an alcohol based spray fixative and air-dried.

Papanicolaou-stained cervical smears (Pap smears) can be screened by the methods of this invention, for example, for retrospective studies. Preferably, Pap smears would be decolorized and re-stained with labeled antibodies against MN antigen. Also archival specimens, for example, matched smears and biopsy and/or tumor specimens, can be used for retrospective studies. Prospective studies can also be done with matched specimens from patients that have a higher than normal risk of exhibiting abnormal cervical cytopathology.

Preferred samples in which to assay MN antigen by, for example, Western blotting or radioimmunoassay, are tissue and/or cell extracts. However, MN antigen may be detected in body fluids, which can include among other fluids: blood, serum, plasma, semen, breast exudate, saliva, tears, sputum, mucous, urine, lymph, cytosols, ascites, pleural effusions, amniotic fluid, bladder washes, bronchioalveolar lavages and cerebrospinal fluid. It is preferred that the MN antigen be concentrated from a larger volume of body fluid before testing. Preferred body fluids to assay would depend on the type of cancer for which one

was testing, but in general preferred body fluids would be breast exudate, pleural effusions and ascites.

MN-specific antibodies can be bound by serologically active MN proteins/polypeptides in samples of such body fluids as blood, plasma, serum, lymph, mucous, tears, urine, spinal fluid and saliva; however, such antibodies are found most usually in blood, plasma and serum, preferably in serum. A representative assay to detect MN-specific antibodies is shown in Example 8 below wherein the fusion protein GEX-3X-MN is used. Correlation of the results from the assays to detect and/or quantitate MN antigen and MN-specific antibodies reactive therewith, provides a preferred profile of the disease condition of a patient.

The assays of this invention are both diagnostic and/or prognostic, i.e., diagnostic/prognostic. The term "diagnostic/ prognostic" is herein defined to encompass the following processes either individually or cumulatively depending upon the clinical context: determining the presence of disease, determining the nature of a disease, distinguishing one disease from another, forecasting as to the probable outcome of a disease state, determining the prospect as to recovery from a disease as indicated by the nature and symptoms of a case, monitoring the disease status

of a patient, monitoring a patient for recurrence of disease, and/or determining the preferred therapeutic regimen for a patient. The diagnostic/prognostic methods of this invention are useful, for example, for screening
5 populations for the presence of neoplastic or pre-neoplastic disease, determining the risk of developing neoplastic disease, diagnosing the presence of neoplastic and/or pre-neoplastic disease, monitoring the disease status of patients with neoplastic disease, and/or determining the
10 prognosis for the course of neoplastic disease. For example, it appears that the intensity of the immunostaining with MN-specific antibodies may correlate with the severity of dysplasia present in samples tested.

The present invention is useful for screening for
15 the presence of a wide variety of neoplastic diseases including carcinomas, such as, mammary, urinary tract, ovarian, uterine, cervical, endometrial, squamous cell and adenosquamous carcinomas; head and neck cancers; mesodermal tumors, such as, neuroblastomas and retinoblastomas;
20 sarcomas, such as osteosarcomas and Ewing's sarcoma; and melanomas. Of particular interest are gynecological cancers including ovarian, uterine, cervical, vaginal, vulval and endometrial cancers, particularly ovarian, uterine cervical and endometrial cancers. Also of particular interest are

cancers of the breast, of the stomach including esophagus, of the colon, of the kidney, of the prostate, of the liver, of the urinary tract including bladder, of the lung, and of the head and neck.

5 The invention provides methods and compositions for evaluating the probability of the presence of malignant or pre-malignant cells, for example, in a group of cells freshly removed from a host. Such an assay can be used to detect tumors, quantitate their growth, and help in the
10 diagnosis and prognosis of disease. The assays can also be used to detect the presence of cancer metastasis, as well as confirm the absence or removal of all tumor tissue following surgery, cancer chemotherapy and/or radiation therapy. It can further be used to monitor cancer chemotherapy and tumor
15 reappearance.

 The presence of MN antigen or antibodies can be detected and/or quantitated using a number of well-defined diagnostic assays. Those in the art can adapt any of the conventional immunoassay formats to detect and/or quantitate
20 MN antigen and/or antibodies. Example 8 details the format of a preferred diagnostic method of this invention--a radioimmunoassay. Immunohistochemical staining is another preferred assay format as exemplified in Example 13.

Many other formats for detection of MN antigen and MN-specific antibodies are, of course available. Those can be Western blots, ELISAs (enzyme-linked immunosorbent assays), RIAs (radioimmunoassay), competitive EIA or dual
5 antibody sandwich assays, among other assays all commonly used in the diagnostic industry. In such immunoassays, the interpretation of the results is based on the assumption that the antibody or antibody combination will not cross-react with other proteins and protein fragments
10 present in the sample that are unrelated to MN.

Representative of one type of ELISA test for MN antigen is a format wherein a microtiter plate is coated with antibodies made to MN proteins/polypeptides or antibodies made to whole cells expressing MN proteins, and
15 to this is added a patient sample, for example, a tissue or cell extract. After a period of incubation permitting any antigen to bind to the antibodies, the plate is washed and another set of anti-MN antibodies which are linked to an enzyme is added, incubated to allow reaction to take place,
20 and the plate is then rewashed. Thereafter, enzyme substrate is added to the microtiter plate and incubated for a period of time to allow the enzyme to work on the substrate, and the adsorbance of the final preparation is

measured. A large change in absorbance indicates a positive result.

It is also apparent to one skilled in the art of immunoassays that MN proteins and/or polypeptides can be
5 used to detect and/or quantitate the presence of MN antigen in the body fluids, tissues and/or cells of patients. In one such embodiment, a competition immunoassay is used, wherein the MN protein/polypeptide is labeled and a body fluid is added to compete the binding of the labeled MN
10 protein/polypeptide to antibodies specific to MN protein/polypeptide. Such an assay can be used to detect and/or quantitate MN antigen as described in Example 8.

In another embodiment, an immunometric assay may be used wherein a labeled antibody made to a MN protein or
15 polypeptide is used. In such an assay, the amount of labeled antibody which complexes with the antigen-bound antibody is directly proportional to the amount of MN antigen in the sample.

A representative assay to detect MN-specific
20 antibodies is a competition assay in which labeled MN protein/polypeptide is precipitated by antibodies in a sample, for example, in combination with monoclonal antibodies recognizing MN proteins/polypeptides. One skilled in the art could adapt any of the conventional

immunoassay formats to detect and/or quantitate MN-specific antibodies. Detection of the binding of said antibodies to said MN protein/polypeptide could be by many ways known to those in the art, e.g., in humans with the use of anti-human
5 labeled IgG.

An exemplary immunoassay method of this invention to detect and/or quantitate MN antigen in a vertebrate sample comprises the steps of:

a) incubating said vertebrate sample with one or
10 more sets of antibodies (an antibody or antibodies) that bind to MN antigen wherein one set is labeled or otherwise detectable;

b) examining the incubated sample for the presence of immune complexes comprising MN antigen and said
15 antibodies.

Another exemplary immunoassay method according to this invention is that wherein a competition immunoassay is used to detect and/or quantitate MN antigen in a vertebrate sample and wherein said method comprises the steps of:

20 a) incubating a vertebrate sample with one or more sets of MN-specific antibodies and a certain amount of a labeled or otherwise detectable MN protein/polypeptide wherein said MN protein/ polypeptide competes for binding to said antibodies with MN antigen present in the sample;

b) examining the incubated sample to determine the amount of labeled/detectable MN protein/polypeptide bound to said antibodies; and

5 c) determining from the results of the examination in step b) whether MN antigen is present in said sample and/or the amount of MN antigen present in said sample.

Once antibodies (including biologically active antibody fragments) having suitable specificity have been prepared, a wide variety of immunological assay methods are
10 available for determining the formation of specific antibody-antigen complexes. Numerous competitive and non-competitive protein binding assays have been described in the scientific and patent literature, and a large number of such assays are commercially available. Exemplary
15 immunoassays which are suitable for detecting a serum antigen include those described in U.S. Patent Nos.

3,791,932; 3,817,837; 3,839,153; 3,850,752; 3,850,578;
3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074;
3,984,533; 3,996,345; 4,034,074; and 4,098,876.

20 Antibodies employed in assays may be labeled or unlabeled. Unlabeled antibodies may be employed in agglutination; labeled antibodies may be employed in a wide variety of assays, employing a wide variety of labels.

Suitable detection means include the use of labels such as radionuclides, enzymes, coenzymes, fluorescers, chemiluminescers, chromogens, enzyme substrates or co-factors, enzyme inhibitors, free radicals, particles, dyes and the like. Such labeled reagents may be used in a variety of well known assays, such as radioimmunoassays, enzyme immunoassays, e.g., ELISA, fluorescent immunoassays, and the like. See for example, U.S. Patent Nos. 3,766,162; 3,791,932; 3,817,837; and 4,233,402.

Methods to prepare antibodies useful in the assays of the invention are described below. The examples below detail representative assays according to this invention.

Immunoassay Test Kits

The above outlined assays can be embodied in test kits to detect and/or quantitate MN antigen and/or MN-specific antibodies (including biologically active antibody fragments). Kits to detect and/or quantitate MN antigen can comprise MN protein(s)/polypeptides(s) and/or MN-specific antibodies, polyclonal and/or monoclonal. Such diagnostic/prognostic test kits can comprise one or more sets of antibodies, polyclonal and/or monoclonal, for a sandwich format wherein antibodies recognize epitopes on the

MN antigen, and one set is appropriately labeled or is otherwise detectable.

Test kits for an assay format wherein there is competition between a labeled (or otherwise detectable) MN
5 protein/polypeptide and MN antigen in the sample, for binding to an antibody, can comprise the combination of the labeled protein/polypeptide and the antibody in amounts which provide for optimum sensitivity and accuracy.

Test kits for MN-specific antibodies preferably
10 comprise labeled/detectable MN proteins(s) and/or polypeptides(s), and may comprise other components as necessary, for example, to perform a preferred assay as outlined in Example 8 below, such as, controls, buffers, diluents and detergents. Such test kits can have other
15 appropriate formats for conventional assays.

A kit for use in an enzyme-immunoassay typically includes an enzyme-labelled reagent and a substrate for the enzyme. The enzyme can, for example, bind either an MN-specific antibody of this invention or to an antibody to
20 such an MN-specific antibody.

Preparation of MN-Specific Antibodies

The term "antibodies" is defined herein to include not only whole antibodies but also biologically active

fragments of antibodies, preferably fragments containing the antigen binding regions. Such antibodies may be prepared by conventional methodology and/or by genetic engineering. Antibody fragments may be genetically engineered, preferably

5 from the variable regions of the light and/or heavy chains (V_H and V_L), including the hypervariable regions, and still more preferably from both the V_H and V_L regions. For example, the term "antibodies" as used herein comprehends polyclonal and monoclonal antibodies and biologically active

10 fragments thereof including among other possibilities "univalent" antibodies [Glennie et al., Nature, 295: 712 (1982)]; Fab proteins including Fab' and $F(ab')_2$ fragments whether covalently or non-covalently aggregated; light or heavy chains alone, preferably variable heavy and light

15 chain regions (V_H and V_L regions), and more preferably including the hypervariable regions [otherwise known as the complementarity determining regions (CDRs) of said V_H and V_L regions]; F_c proteins; "hybrid" antibodies capable of binding more than one antigen; constant-variable region

20 chimeras; "composite" immunoglobulins with heavy and light chains of different origins; "altered" antibodies with improved specificity and other characteristics as prepared by standard recombinant techniques and also by

oligonucleotide-directed mutagenesis techniques [Dalbadie-McFarland et al., PNAS (USA), 79: 6409 (1982)].

It may be preferred for therapeutic and/or imaging uses that the antibodies be biologically active antibody
5 fragments, preferably genetically engineered fragments, more preferably genetically engineered fragments from the V_H and/or V_L regions, and still more preferably comprising the hypervariable regions thereof.

There are conventional techniques for making
10 polyclonal and monoclonal antibodies well-known in the immunoassay art. Immunogens to prepare MN-specific antibodies include MN proteins and/or polypeptides, preferably purified, and MX-infected tumor line cells, for example, MX-infected HeLa cells, among other immunogens.

15 Anti-peptide antibodies are also made by conventional methods in the art as described in European Patent Publication No. 44,710 (published Jan. 27, 1982). Briefly, such anti-peptide antibodies are prepared by selecting a peptide from an MN amino acid sequence as from
20 Figures 1A-1C, chemically synthesizing it, conjugating it to an appropriate immunogenic protein and injecting it into an appropriate animal, usually a rabbit or a mouse; then, either polyclonal or monoclonal antibodies are made, the latter by a Kohler-Milstein procedure, for example.

Besides conventional hybridoma technology, newer technologies can be used to produce antibodies according to this invention. For example, the use of the PCR to clone and express antibody V-genes and phage display technology to
5 select antibody genes encoding fragments with binding activities has resulted in the isolation of antibody fragments from repertoires of PCR amplified V-genes using immunized mice or humans. [Marks et al., BioTechnology, 10: 779 (July 1992) for references; Chiang et al.,
10 BioTechniques, 7(4): 360 (1989); Ward et al., Nature, 341: 544 (Oct. 12, 1989); Marks et al., J. Mol. Biol., 222: 581 (1991); Clackson et al., Nature, 352: (15 August 1991); and Mullinax et al., PNAS (USA), 87: 8095 (Oct. 1990).]

Descriptions of preparing antibodies, which term
15 is herein defined to include biologically active antibody fragments, by recombinant techniques can be found in U.S. Patent No. 4,816,567 (issued March 28, 1989); European Patent Application Publication Number (EP) 338,745 (published Oct. 25, 1989); EP 368,684 (published June 16,
20 1990); EP 239,400 (published September 30, 1987); WO 90/14424 (published Nov. 29, 1990); WO 90/14430 (published May 16, 1990); Huse et al., Science, 246: 1275 (Dec. 8, 1989); Marks et al., BioTechnology, 10: 779 (July 1992); La Sastry et al., PNAS (USA), 86: 5728 (August 1989); Chiang

et al., BioTechniques, 7(40): 360 (1989); Orlandi et al.,
PNAS (USA), 86: 3833 (May 1989); Ward et al. Nature, 341:
544 (October 12, 1989); Marks et al., J. Mol. Biol., 222:
581 (1991); and Hoogenboom et al., Nucleic Acids Res.,
5 19(15): 4133 (1991).

Representative Mabs

Monoclonal antibodies for use in the assays of
this invention may be obtained by methods well known in the
art for example, Galfre and Milstein, "Preparation of
10 Monoclonal Antibodies: Strategies and Procedures," in
Methods in Enzymology: Immunochemical Techniques, 73: 1-46
[Langone and Vanatis (eds); Academic Press (1981)]; and in
the classic reference, Milstein and Kohler, Nature, 256:
495-497 (1975).]

15 Although representative hybridomas of this
invention are formed by the fusion of murine cell lines,
human/human hybridomas [Olsson et al., PNAS (USA), 77: 5429
(1980)] and human/murine hybridomas [Schlom et al., PNAS
(USA), 77: 6841 (1980); Shearman et al. J. Immunol., 146:
20 928-935 (1991); and Gorman et al., PNAS (USA), 88: 4181-
4185 (1991)] can also be prepared among other possibilities.
Such humanized monoclonal antibodies would be preferred
monoclonal antibodies for therapeutic and imaging uses.

Monoclonal antibodies specific for this invention can be prepared by immunizing appropriate mammals, preferably rodents, more preferably rabbits or mice, with an appropriate immunogen, for example, MaTu-infected HeLa
5 cells, MN fusion proteins, or MN proteins/polypeptides attached to a carrier protein if necessary. Exemplary methods of producing antibodies of this invention are described below.

The monoclonal antibodies useful according to this
10 invention to identify MN proteins/polypeptides can be labeled in any conventional manner, for example, with enzymes such as horseradish peroxidase (HRP), fluorescent compounds, or with radioactive isotopes such as, ^{125}I , among other labels. A preferred label, according to this
15 invention is ^{125}I , and a preferred method of labeling the antibodies is by using chloramine-T [Hunter, W.M., "Radioimmunoassay," In: Handbook of Experimental Immunology, pp. 14.1-14.40 (D.W. Weir ed.; Blackwell, Oxford/London/Edinburgh/Melbourne; 1978)].

20 Representative mabs of this invention include Mabs M75, MN9, MN12 and MN7 described below. Monoclonal antibodies of this invention serve to identify MN proteins/polypeptides in various laboratory diagnostic

tests, for example, in tumor cell cultures or in clinical samples.

Mabs Prepared Against HeLa Cells

5 MAb M75. Monoclonal antibody M75 (MAb M75) is produced by mouse lymphocytic hybridoma VU-M75, which was initially deposited in the Collection of Hybridomas at the Institute of Virology, Slovak Academy of Sciences (Bratislava, Slovak Republic) and was deposited under ATCC Designation HB 11128 on September 17, 1992 at the American
10 Type Culture Collection (ATCC) in Manassas, Virginia (USA).

Hybridoma VU-M75 was produced according to the procedure described in Gerhard, W., "Fusion of cells in suspension and outgrowth of hybrids in conditioned medium,"
In: Monoclonal Antibodies. Hybridomas: A New Dimension in
15 Biological Analysis, page 370 [Kennet et al. (eds.); Plenum NY (USA)]. BALB/C mice were immunized with MaTu-infected HeLa cells, and their spleen cells were fused with myeloma cell line NS-0. Tissue culture media from the hybridomas were screened for monoclonal antibodies, using as antigen
20 the p58 immunoprecipitated from cell extracts of MaTu-infected HeLa with rabbit anti-MaTu serum and protein A-
Staphylococcus aureus cells (SAC) [Zavada and Zavadova, Arch. Virol., 118 189-197 (1991)], and eluted from SDS-PAGE

gels. Monoclonal antibodies were purified from TC media by affinity chromatography on protein A-Sepharose [Harlow and Lane, "Antibodies: A Laboratory Manual," Cold Spring Harbor, Cold Spring Harbor, NY (USA); 1988].

5 Mab M75 recognizes both the nonglycosylated GEX-3X-MN fusion protein and native MN protein as expressed in CGL3 cells equally well. Mab M75 was shown by epitope mapping to be reactive with the epitope represented by the amino acid sequence from AA 62 to AA 67 [SEQ. ID. NO.: 10]
10 of the MN protein shown in Figures 1A-1C.

Mabs M16 and M67. Also produced by the method described for producing MAb M75 (isotype IgG2B) were MAb M16 (isotype IgG2A) and M67 (isotype IgG1). Mabs M16 and M67 recognize MX protein, as described in the examples
15 below.

MAb H460. Monoclonal antibody H460 (MAb H460) was prepared in a manner similar to that for MAb M75 except that the mice were immunized with HeLa cells uninfected with MaTu, and lymphocytes of the mice rather than spleen cells
20 were fused with cells from myeloma cell line NS-0. MAb H460 reacts about equally with any human cells.

Mabs Prepared Against Fusion Protein GEX-3X-MN

Monoclonal antibodies of this invention were also prepared against the MN glutathione S-transferase fusion protein (GEX-3X-MN) purified by affinity chromatography as
5 described above. BALB/C mice were immunized intraperitoneally according to standard procedures with the GEX-3X-MN fusion protein in Freund's adjuvant. Spleen cells of the mice were fused with SP/20 myeloma cells [Milstein and Kohler, supra].

10 Tissue culture media from the hybridomas were screened against CGL3 and CGL1 membrane extracts in an ELISA employing HRP labelled-rabbit anti-mouse. The membrane extracts were coated onto microtiter plates. Selected were antibodies reacted with the CGL3 membrane extract. Selected
15 hybridomas were cloned twice by limiting dilution.

The mabs prepared by the just described method were characterized by Western blots of the GEX-3X-MN fusion protein, and with membrane extracts from the CGL1 and CGL3 cells. Representative of the mabs prepared are Mabs MN9,
20 MN12 and MN7.

Mab MN9. Monoclonal antibody MN9 (Mab MN9) reacts to the same epitope as Mab M75, represented by the sequence from AA 62 to AA 67 [SEQ. ID. NO.: 10] of the Figures 1A-1C

MN protein. As Mab M75, Mab MN9 recognizes both the GEX-3X-MN fusion protein and native MN protein equally well.

Mabs corresponding to Mab MN9 can be prepared reproducibly by screening a series of mabs prepared against
5 an MN protein/polypeptide, such as, the GEX-3X-MN fusion protein, against the peptide representing the epitope for Mabs M75 and MN9, that is, SEQ. ID. NO.: 10.

Alternatively, the Novatope system [Novagen] or competition with the deposited Mab M75 could be used to select mabs
10 comparable to Mabs M75 and MN9.

Mab MN12. Monoclonal antibody MN12 (Mab MN12) is produced by the mouse lymphocytic hybridoma MN 12.2.2 which was deposited under ATCC Designation HB 11647 on June 9, 1994 at the American Type Culture Collection (ATCC) at 10801
15 University Blvd., Manassas, Virginia 20110-2209 (USA).

Antibodies corresponding to Mab MN12 can also be made, analogously to the method outlined above for Mab MN9, by screening a series of antibodies prepared against an MN protein/polypeptide, against the peptide representing the
20 epitope for Mab MN12. That peptide is AA 55 - AA 60 of Figures 1A-1C [SEQ. ID. NO.: 11]. The Novatope system could also be used to find antibodies specific for said epitope.

Mab MN7. Monoclonal antibody MN7 (Mab MN7) was selected from mabs prepared against nonglycosylated GEX-3X-MN as described above. It recognizes the epitope on MN represented by the amino acid sequence from AA 127 to AA 147
5 [SEQ. ID. NO.: 12] of the Figures 1A-1C MN protein. Analogously to methods described above for Mabs MN9 and MN12, mabs corresponding to Mab MN7 can be prepared by selecting mabs prepared against an MN protein/polypeptide that are reactive with the peptide having SEQ. ID. NO.: 12,
10 or by the stated alternative means.

Epitope Mapping

Epitope mapping was performed by the Novatope system, a kit for which is commercially available from Novagen, Inc. [See, for analogous example, Li et al.,
15 Nature, 363: 85-88 (6 May 1993).] In brief, the MN cDNA was cut into overlapping short fragments of approximately 60 base pairs. The fragments were expressed in E. coli, and the E. coli colonies were transferred onto nitrocellulose paper, lysed and probed with the mab of interest. The MN
20 cDNA of clones reactive with the mab of interest was sequenced, and the epitopes of the mabs were deduced from the overlapping polypeptides found to be reactive with each mab.

Therapeutic Use of MN-Specific Antibodies

The MN-specific antibodies of this invention, monoclonal and/or polyclonal, preferably monoclonal, and as outlined above, may be used therapeutically in the treatment of neoplastic and/or pre-neoplastic disease, either alone or
5 in combination with chemotherapeutic drugs or toxic agents, such as ricin A. Further preferred for therapeutic use would be biologically active antibody fragments as described herein. Also preferred MN-specific antibodies for such
10 therapeutic uses would be humanized monoclonal antibodies.

The MN-specific antibodies can be administered in a therapeutically effective amount, preferably dispersed in a physiologically acceptable, nontoxic liquid vehicle.

Imaging Use of Antibodies

15 Further, the MN-specific antibodies of this invention when linked to an imaging agent, such as a radionuclide, can be used for imaging. Biologically active antibody fragments or humanized monoclonal antibodies, may be preferred for imaging use.

20 A patient's neoplastic tissue can be identified as, for example, sites of transformed stem cells, of tumors and locations of any metastases. Antibodies, appropriately labeled or linked to an imaging agent, can be injected in a

physiologically acceptable carrier into a patient, and the binding of the antibodies can be detected by a method appropriate to the label or imaging agent, for example, by scintigraphy.

5 Antisense MN Nucleic Acid Sequences

MN genes are herein considered putative oncogenes and the encoded proteins thereby are considered to be putative oncoproteins. Antisense nucleic acid sequences substantially complementary to mRNA transcribed from MN
10 genes, as represented by the antisense oligodeoxynucleotides (ODNs) of Example 10, infra, can be used to reduce or prevent expression of the MN gene. [Zamecnick, P.C., "Introduction: Oligonucleotide Base Hybridization as a Modulator of Genetic Message Readout," pp. 1-6, Prospects
15 for Antisense Nucleic Acid Therapy of Cancer and AIDS, (Wiley-Liss, Inc., New York, NY, USA; 1991); Wickstrom, E., "Antisense DNA Treatment of HL-60 Promyelocytic Leukemia Cells: Terminal Differentiation and Dependence on Target Sequence," pp. 7-24, id.; Leserman et al., "Targeting and
20 Intracellular Delivery of Antisense Oligonucleotides Interfering with Oncogene Expression," pp. 25-34, id.; Yokoyama, K., "Transcriptional Regulation of c-myc Proto-oncogene by Antisense RNA," pp. 35-52, id.; van den Berg et

- al., "Antisense fos Oligodeoxyribonucleotides Suppress the Generation of Chromosomal Aberrations," pp. 63-70, id.;
- Mercola, D., "Antisense fos and fun RNA," pp. 83-114, id.;
- Inouye, Gene, 72: 25-34 (1988); Miller and Ts'o, Ann. Reports Med. Chem., 23: 295-304 (1988); Stein and Cohen, Cancer Res., 48: 2659-2668 (1988); Stevenson and Inversen, J. Gen. Virol., 70: 2673-2682 (1989); Goodchild, "Inhibition of Gene Expression by Oligonucleotides," pp. 53-77, Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression (Cohen, J.S., ed; CRC Press, Boca Raton, Florida, USA; 1989); Dervan et al., "Oligonucleotide Recognition of Double-helical DNA by Triple-helix Formation," pp. 197-210, id.; Neckers, L.M., "Antisense Oligodeoxynucleotides as a Tool for Studying Cell Regulation: Mechanisms of Uptake and Application to the Study of Oncogene Function," pp. 211-232, id.; Leitner et al., PNAS (USA), 87: 3430-3434 (1990); Bevilacqua et al., PNAS (USA), 85: 831-835 (1988); Loke et al. Curr. Top. Microbiol. Immunol., 141: 282-288 (1988); Sarin et al., PNAS (USA), 85: 7448-7451 (1988); Agrawal et al., "Antisense Oligonucleotides: A Possible Approach for Chemotherapy and AIDS," International Union of Biochemistry Conference on Nucleic Acid Therapeutics (Jan. 13-17, 1991; Clearwater Beach, Florida, USA); Armstrong, L., Ber. Week, pp. 88-89 (March 5, 1990); and Weintraub et al., Trends, 1:

22-25 (1985).] Such antisense nucleic acid sequences, preferably oligonucleotides, by hybridizing to the MN mRNA, particularly in the vicinity of the ribosome binding site and translation initiation point, inhibits translation of the mRNA. Thus, the use of such antisense nucleic acid sequences may be considered to be a form of cancer therapy.

Preferred antisense oligonucleotides according to this invention are gene-specific ODNs or oligonucleotides complementary to the 5' end of MN mRNA. Particularly preferred are the 29-mer ODN1 and 19-mer ODN2 for which the sequences are provided in Example 10, infra. Those antisense ODNs are representative of the many antisense nucleic acid sequences that can function to inhibit MN gene expression. Ones of ordinary skill in the art could determine appropriate antisense nucleic acid sequences, preferably antisense oligonucleotides, from the nucleic acid sequences of Figures 1A-1C and 15A-15F.

Also, as described above, CGL3 cells transfected with an "antisense" MN cDNA/promoter construct formed colonies much smaller than control CGL3 cells.

Vaccines

It will be readily appreciated that MN proteins and polypeptides of this invention can be incorporated into

vaccines capable of inducing protective immunity against neoplastic disease and a dampening effect upon tumorigenic activity. Efficacy of a representative MN fusion protein GEX-3X-MN as a vaccine in a rat model is shown in Example
5 14.

MN proteins and/or polypeptides may be synthesized or prepared recombinantly or otherwise biologically, to comprise one or more amino acid sequences corresponding to one or more epitopes of the MN proteins either in monomeric
10 or multimeric form. Those proteins and/or polypeptides may then be incorporated into vaccines capable of inducing protective immunity. Techniques for enhancing the antigenicity of such polypeptides include incorporation into a multimeric structure, binding to a highly immunogenic
15 protein carrier, for example, keyhole limpet hemocyanin (KLH), or diphtheria toxoid, and administration in combination with adjuvants or any other enhancers of immune response.

Preferred MN proteins/polypeptides to be used in a
20 vaccine according to this invention would be genetically engineered MN proteins. Preferred recombinant MN protein are the GEX-3X-MN, MN 20-19, MN-Fc and MN-PA proteins.

Other exemplary vaccines include vaccinia-MN (live vaccinia virus with full-length MN cDNA), and baculovirus-MN

(full length MN cDNA inserted into baculovirus vector, e.g. in suspension of infected insect cells). Different vaccines may be combined and vaccination periods can be prolonged.

5 A preferred exemplary use of such a vaccine of this invention would be its administration to patients whose MN-carrying primary cancer had been surgically removed. The vaccine may induce active immunity in the patients and prevent recidivism or metastasis.

10 It will further be appreciated that anti-idiotypic antibodies to antibodies to MN proteins/polypeptides are also useful as vaccines and can be similarly formulated.

An amino acid sequence corresponding to an epitope of an MN protein/polypeptide either in monomeric or multimeric form may also be obtained by chemical synthetic means or by purification from biological sources including
15 genetically modified microorganisms or their culture media. [See Lerner, "Synthetic Vaccines", Sci. Am. 248(2): 66-74 (1983).] The protein/polypeptide may be combined in an amino acid sequence with other proteins/polypeptides
20 including fragments of other proteins, as for example, when synthesized as a fusion protein, or linked to other antigenic or non-antigenic polypeptides of synthetic or biological origin. In some instances, it may be desirable to fuse a MN protein or polypeptide to an immunogenic and/or

antigenic protein or polypeptide, for example, to stimulate efficacy of a MN-based vaccine.

The term "corresponding to an epitope of an MN protein/polypeptide" will be understood to include the practical possibility that, in some instances, amino acid sequence variations of a naturally occurring protein or polypeptide may be antigenic and confer protective immunity against neoplastic disease and/or anti-tumorigenic effects. Possible sequence variations include, without limitation, amino acid substitutions, extensions, deletions, truncations, interpolations and combinations thereof. Such variations fall within the contemplated scope of the invention provided the protein or polypeptide containing them is immunogenic and antibodies elicited by such a polypeptide or protein cross-react with naturally occurring MN proteins and polypeptides to a sufficient extent to provide protective immunity and/or anti-tumorigenic activity when administered as a vaccine.

Such vaccine compositions will be combined with a physiologically acceptable medium, including immunologically acceptable diluents and carriers as well as commonly employed adjuvants such as Freund's Complete Adjuvant, saponin, alum, and the like. Administration would be in immunologically effective amounts of the MN proteins or

polypeptides, preferably in quantities providing unit doses of from 0.01 to 10.0 micrograms of immunologically active MN protein and/or polypeptide per kilogram of the recipient's body weight. Total protective doses may range from 0.1 to
5 about 100 micrograms of antigen.

Routes of administration, antigen dose, number and frequency of injections are all matters of optimization within the scope of the ordinary skill in the art.

The following examples are for purposes of
10 illustration only and not meant to limit the invention in any way.

Materials and Methods

The following materials and methods were used in examples below.

15 MaTu-Infected and Uninfected HeLa Cells

MaTu agent [Zavada et al., Nature New Biol., 240: 124-125 (1972); Zavada et al., J. Gen. Virol, 24: 327-337 (1974)] was from original "MaTu" cells [Widmaier et al., Arch. Geschwulstforsch, 44: 1-10 (1974)] transferred into
20 our stock of HeLa by cocultivation with MaTu cells treated with mitomycin C, to ensure that control and MaTu-infected cells were comparable. MaTu cells were incubated for 3

hours at 37°C in media with 5 µg/ml of mitomycin C
[Calbiochem; LaJolla, CA (USA)]. Mixed cultures were set to
2 x 10⁵ of mitomycin C-treated cells and 4 x 10⁵ of fresh
recipient cells in 5 ml of medium. After 3 days they were
5 first subcultured and further passaged 1-2 times weekly.

Control HeLa cells were the same as those
described in Zavada et al. (1972), supra.

Sera

Human sera from cancer patients, from patients
10 suffering with various non-tumor complaints and from healthy
women were obtained from the Clinics of Obstetrics and
Gynaecology at the Postgraduate Medical School, Bratislava,
Slovak Republic. Human serum KH was from a fifty year old
mammary carcinoma patient, fourteen months after resection.
15 That serum was one of two sera out of 401 serum samples that
contained neutralizing antibodies to the VSV(MaTu)
pseudotype as described in Zavada et al. (1972), supra.
Serum L8 was from a patient with Paget's disease. Serum M7
was from a healthy donor.

20 Rabbit anti-MaTu serum was prepared by immunizing
a rabbit three times at intervals of 30 days with 10⁻⁵ x 10⁷
viable MaTu-infected HeLa cells.

RIP and PAGE

RIP and PAGE were performed essentially as described in Zavada and Zavadova, supra, except that in the experiments described herein [³⁵S]methionine (NEN), 10
5 μCi/ml of methionine-free MEM medium, supplemented with 2% FCS and 3% complete MEM were used. Confluent petri dish cultures of cells were incubated overnight in that media.

For RIP, the SAC procedure [Kessler, J. Immunol., 115: 1617-1624 (1975)] was used. All incubations and
10 centrifugations were performed at 0-4°C. Cell monolayers were extracted with RIPA buffer (0.14 M NaCl, 7.5 mM phosphate buffer, pH 7.2, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and Trasylol). To reduce non-specific reactions, antisera were
15 preabsorbed with fetal calf serum [Barbacid et al., PNAS (USA), 77: 1617-1621 (1980)] and antigenic extracts with SAC.

For PAGE (under reducing conditions) we used 10% gels with SDS [Laemmli, Nature, 227: 680-685 (1970)]. As
20 reference marker proteins served the Sigma kit [product MW-SDS-200; St. Louis, MO (USA)]. For fluorography we used salicylate [Heegaard et al., Electrophoresis, 5: 263-269 (1984)].

Immunoblots

Immunoblotting used as described herein follows the method of Towbin et al., PNAS (USA), 76: 4350-4354 (1979). The proteins were transferred from the gels onto
5 nitrocellulose [Schleicher and Schuell; Dassel Germany; 0.45 μ m porosity] in Laemmli electrode buffer diluted 1:10 with distilled water, with no methanol or SDS. The transfer was for 2 1/2 hours at 1.75 mA/cm². The blots were developed with ¹²⁵I-labeled MAbs and autoradiography was performed
10 using intensifying screens, with X-ray films exposed at - 70°C.

In extracts from cell cultures containing only small amounts of MN antigen, we concentrated the antigen from 0.5 or 1 ml of an extract by adding 50 μ l of a 10% SAC
15 suspension, pre-loaded with MAb M75. This method allowed the concentration of MN antigen even from clinical specimens, containing human IgG; preliminary control experiments showed that such a method did not interfere with the binding of the MN antigen to SAC-adsorbed M75. Tissue
20 extracts were made by grinding the tissue with a mortar and pestle and sand (analytical grade). To the homogenates was added RIPA buffer, 10:1 (volume to weight) of original tissue. The extracts were clarified for 3 minutes on an Eppendorf centrifuge.

Example 1

Immunofluorescence of MaTu-Specific Antigens

Immunofluorescence experiments were performed on control and MaTu-infected HeLa cells with monoclonal
5 antibodies, prepared as described above, which are specific for MaTu-related antigens. FITC-conjugated anti-mouse IgG was used to detect the presence of the monoclonal antibodies. Staining of the cells with Giemsa revealed no clear differences between control and MaTu-infected HeLa
10 cells.

MABs, which in preliminary tests proved to be specific for MaTu-related antigens, showed two different reactivities in immunofluorescence. A representative of the first group, MAB M67, gave a granular cytoplasmic
15 fluorescence in MaTu-infected HeLa, which was only seen in cells fixed with acetone; living cells showed no fluorescence. MAB M16 gave the same type of fluorescence. With either M67 or M16, only extremely weak "background" fluorescence was seen in control HeLa cells.

20 Another MAB, M75, showed a granular membrane fluorescence on living MaTu-infected cells and a granular nuclear fluorescence in acetone-fixed cells. However, M75 sometimes showed a similar, although much weaker, fluorescence on uninfected HeLa cells. A relationship was

observed based upon the conditions of growth: in HeLa cells uninfected with MaTu, both types of fluorescence with MAb M75 were observed only if the cells were grown for several passages in dense cultures, but not in sparse ones.

5 The amount of M75-reactive cell surface antigen was analyzed cytofluorometrically and was dependent on the density of the cell cultures and on infection with MaTu. Control and MaTu infected HeLa cells were grown for 12 days in dense or sparse cultures. The cells were released with
10 Versene (EDTA), and incubated with MAb M75 or with no MAb, and subsequently incubated with FITC-conjugated anti-mouse IgG. The intensity of fluorescence was measured.

 It appeared that the antigen binding MAb M75 is inducible: it was found to be absent in control HeLa grown
15 in sparse culture, and to be induced either by the growth of HeLa in dense culture or by infection with MaTu. Those two factors were found to have an additive or synergistic effect. Those observations indicated along with other results described herein that there were two different
20 agents involved: exogenous, transmissible MX, reactive with M67, and endogenous, inducible MN, detected by MAb M75.

Example 2

Immunoblot Analysis of Protein(s) Reactive with MAb M75

To determine whether MAb M75 reacts with the same protein in both uninfected and MaTu-infected HeLa, and to
5 determine the molecular weight of the protein, extracts of those cells were analyzed by PAGE and immunoblotting (as described above). HeLa cells uninfected or MaTu-infected, that had been grown for 12 days in dense or sparse cultures, were seeded in 5-cm petri dishes, all variants at 5×10^5
10 cells per dish. Two days later, the cells were extracted with RIPA buffer (above described), 200 μ l/dish. The extracts were mixed with 2x concentrated Laemmli sample buffer containing 6% mercaptoethanol and boiled for five minutes. Proteins were separated by SDS-PAGE and blotted on
15 nitrocellulose. The blots were developed with 125 I-labeled MAb M75 and autoradiography.

MAb M75 reacted with two MN-specific protein bands of 54 kd and 58 kd, which were the same in uninfected HeLa grown at high density and in MaTu-infected HeLa, evidencing
20 that M75 recognizes the same protein(s) in both uninfected and MaTu-infected HeLa cells. Consistent with the cytofluorometric results, the amount of the antigen depended both on cell density and on infection with MaTu, the latter being a much more potent inducer of p54/58N.

Example 3

Radioimmunoassay of MaTu-Specific Antigens In Situ

In contrast to the results with M75, the other MAb, M67, appeared to be specific for the exogenous,
5 transmissible agent MX. With M67 we observed no immunofluorescence in control HeLa, regardless of whether the cells were grown in dense or in sparse culture. That difference was clearly evidenced in radioimmunoassay experiments wherein ^{125}I -labeled MAbs M67 and M75 were used.

10 For such experiments, parallel cultures of uninfected and MaTu-infected cells were grown in dense or sparse cultures. The cultures were either live (without fixation), or fixed (with methanol for five minutes and air-dried). The cultures were incubated for two hours in petri
15 dishes with the ^{125}I -labeled MAbs, 6×10^4 cpm/dish. Afterward, the cultures were rinsed four times with PBS and solubilized with 1 ml/dish of 2 N NaOH, and the radioactivity was determined on a gamma counter.

The simple radioimmunoassay procedure of this
20 example was performed directly in petri dish cultures. Sixteen variants of the radioimmunoassay enabled us to determine whether the MX and MN antigens are located on the surface or in the interior of the cells and how the expression of those two antigens depends on infection with

MaTu and on the density, in which the cells had been grown before the petri dishes were seeded. In live, unfixed cells only cell surface antigens can bind the MAbs. In those cells, M67 showed no reaction with any variant of the cultures, whereas M75 reacted in accord with the results of Examples 1 and 2 above.

Fixation of the cells with methanol made the cell membrane permeable to the MAbs: M67 reacted with HeLa infected with MaTu, independently of previous cell density, and it did not bind to control HeLa. MAb M75 in methanol-fixed cells confirmed the absence of corresponding antigen in uninfected HeLa from sparse cultures and its induction both by growth in dense cultures and by infection with MaTu.

Example 4

Identification of MaTu Components Reactive with Animal Sera or Associated with VSV Virions

Immunoblot analyses of MaTu-specific proteins from RIPA extracts from uninfected or MaTu-infected HeLa and from purified VSV reproduced in control or in MaTu-infected HeLa, identified which of the antigens, p58X or p54/58N, were radioimmunoprecipitated with animal sera, and which of them was responsible for complementation of VSV mutants and for the formation of pseudotype virions. Details concerning the

procedures can be found in Pastorekova et al., Virology,
187: 620-626 (1992).

5 The serum of a rabbit immunized with MaTu-infected
HeLa immunoprecipitated both MAb M67- and MAb M75-reactive
proteins (both p58X and p54/58N), whereas the
"spontaneously" immune sera of normal rabbit, sheep or
leukemic cow immunoprecipitated only the M67-reactive
protein (p58X). On the other hand, in VSV reproduced in
MaTu-infected HeLa cells and subsequently purified, only the
10 M75-reactive bands of p54/58N were present. Thus, it was
concluded that MX and MN are independent components of MaTu,
and that it was p54/58N that complemented VSV mutants and
was assembled into pseudotype virions.

As shown in Figures 6A and 6B discussed below in
15 Example 5, MX antigen was found to be present in MaTu-
infected fibroblasts. In Zavada and Zavadova, supra, it was
reported that a p58 band from MX-infected fibroblasts could
not be detected by RIP with rabbit anti-MaTu serum. That
serum contains more antibodies to MX than to MN antigen.
20 The discrepancy can be explained by the extremely slow
spread of MX in infected cultures. The results reported in
Zavada and Zavadova, supra were from fibroblasts tested 6
weeks after infection, whereas the later testing was 4
months after infection. We have found by immunoblots that

MX can be first detected in both H/F-N and H/F-T hybrids after 4 weeks, in HeLa cells after six weeks and in fibroblasts only 10 weeks after infection.

Example 5

5 Expression of MN- and MX- Specific Proteins

Figures 6A and 6B graphically illustrate the expression of MN- and MX- specific proteins in human fibroblasts, in HeLa cells and in H/F-N and H/F-T hybrid cells, and contrasts the expression in MX-infected and
10 uninfected cells. Cells were infected with MX by co-cultivation with mitomycin C-treated MX-infected HeLa. The infected and uninfected cells were grown for three passages in dense cultures. About four months after infection, the infected cells concurrently with uninfected cells were grown
15 in petri dishes to produce dense monolayers.

A radioimmunoassay was performed directly in confluent petri dish (5 cm) culture of cells, fixed with methanol essentially as described in Example 3, supra. The monolayers were fixed with methanol and treated with ¹²⁵I-
20 labeled MAbs M67 (specific for exogenous MX antigen) or M75 (specific for endogenous MN antigen) at 6×10^4 cpm/dish. The bound radioactivity was measured; the results are shown in Figures 6A and 6B.

Figures 6A and 6B show that MX was transmitted to all four cell lines tested, that is, to human embryo fibroblasts, to HeLa and to both H/F-N and H/F-T hybrids; at the same time, all four uninfected counterpart cell lines were MX-negative (top graph of Figures 6A and 6B). MN antigens are shown to be present in both MX-infected and uninfected HeLa and H/F-T cells, but not in the fibroblasts (bottom graph of Figures 6A and 6B). No MN antigen was found in the control H/F-N, and only a minimum increase over background of MN antigen was found in MaTu infected H/F-N. Thus, it was found that in the hybrids, expression of MN antigen very strongly correlates with tumorigenicity.

Those results were consistent with the results obtained by immunoblotting as shown in Figure 7. The MN-specific twin protein p54/58N was detected in HeLa cell lines (both our standard type, that is, HeLa K, and in the Stanbridge mutant HeLa, that is, D98/AH.2 shown as HeLa S) and in tumorigenic H/F-T; however, p54/58N was not detected in the fibroblasts nor in the non-tumorigenic H/F-N even upon deliberately long exposure of the film used to detect radioactivity. Infection of the HeLa cells with MX resulted in a strong increase in the concentration of the p54/58N protein(s).

The hybrid cells H/F-N and H/F-T were constructed by Eric J. Stanbridge [Stanbridge et al., Somatic Cell Genetics, 7: 699-712 (1981); and Stanbridge et al., Science, 215: 252-259 (1982)]. His original hybrid, 5 produced by the fusion of a HeLa cell and a human fibroblast was not tumorigenic in nude mice, although it retained some properties of transformed cells, for example, its growth on soft agar. Rare segregants from the hybrid which have lost chromosome 11 are tumorigenic. The most likely explanation 10 for the tumorigenicity of those segregants is that chromosome 11 contains a suppressor gene (an antioncogene), which blocks the expression of a as yet unknown oncogene. The oncoprotein encoded by that oncogene is critical for the capacity of the H/F hybrids to produce tumors in nude mice. 15 Since the p54/58N protein shows a correlation with the tumorigenicity of H/F hybrids, it is a candidate for that putative oncoprotein.

Example 6

Immunoblots of MN Antigen from Human Tumor Cell Cultures and from Clinical Specimens of Human Tissues

 20

The association of MN antigen with tumorigenicity in the H/F hybrid cells as illustrated by Example 5 prompted testing for the presence of MN antigen in other human tumor

cell cultures and in clinical specimens. Preliminary experiments indicated that the concentration of MN antigen in the extracts from other human tumor cell cultures was lower than in HeLa; thus, it was realized that long exposure of the autoradiographs would be required. Therefore, the sensitivity of the method was increased by the method indicated under Materials and Methods: Immunoblotting, supra, wherein the MN antigen was concentrated by precipitation with MAb M75-loaded SAC.

Figure 8 shows the immunoblots wherein lane A, a cell culture extract from MX-infected HeLa cells was analysed directly (10 μ l per lane) whereas the antigens from the other extracts (lanes B-E) were each concentrated from a 500 μ l extract by precipitation with MAb M75 and SAC.

Figure 8 indicates that two other human carcinoma cell lines contain MN-related proteins -- T24 (bladder carcinoma; lane C) and T47D (mammary carcinoma; lane D). Those cells contain proteins which react with MAb M75 that under reducing conditions have molecular weights of 54 kd and 56 kd, and under non-reducing conditions have a molecular weight of about 153 kd. The intensity of those bands is at least ten times lower than that for the p54/58N twin protein from HeLa cells.

An extremely weak band at approximately 52 kd could be seen under reducing conditions from extracts from human melanoma cells (SK-Mel 1477; lane E), but no bands for human fibroblast extracts (lane B) could be seen either on
5 the reducing or non-reducing gels.

Figure 9 shows immunoblots of human tissue extracts including surgical specimens as compared to a cell extract from MX-infected HeLa (lane A). The MN-related antigen from all the extracts but for lane A (analysed
10 directly at 10 μ l per lane) was first concentrated from a 1 ml extract as explained above. MN proteins were found in endometrial (lanes D and M), ovarian (lanes E and N) and in uterine cervical (lane O) carcinomas. In those extracts MN-related proteins were found in three bands having molecular
15 weights between about 48 kd and about 58 kd. Another MN-related protein was present in the tissue extract from a mammary papilloma; that protein was seen as a single band at about 48 kd (lane J).

Clearly negative were the extracts from full-term
20 placenta (lane B), normal mammary gland (lane K), hyperplastic endometrium (lane L), normal ovaries (lane H), and from uterine myoma (lane I). Only extremely slightly MN-related bands were seen in extracts from trophoblasts (lanes F and G) and from melanoma (lane P).

The observations that antigen related to p54/58N was expressed in clinical specimens of several types of human carcinomas but not in general in normal tissues of the corresponding organs (exceptions delineated in Example 13) further strengthened the association of MN antigen with tumorigenesis. However, it should be noted that for human tumors, a normal tissue is never really an adequate control in that tumors are believed not to arise from mature, differentiated cells, but rather from some stem cells, capable of division and of differentiation. In body organs, such cells may be quite rare.

Example 7

MN Antigen in Animal Cell Lines

Since the MN gene is present in the chromosomal DNA of all vertebrate species that were tested, MN-related antigen was searched for also in cell lines derived from normal tissues and from tumors of several animal species. MN-related protein was found in two rat cell lines: one of them was the XC cell line derived from rat rhabdomyosarcoma induced with Rous sarcoma virus; the other was the Rat2-Tk⁻ cell line. In extracts from both of those rat cell lines, a single protein band was found on the blots : its molecular weight on blots produced from a reducing gel and from a non-

reducing gel was respectively 53.5 kd and 153 kd. Figure 10 shows the results with Rat2-Tk⁻ cell extracts (lane B), compared with extracts from MX-infected HeLa (lane A); the concentration of MN antigen in those two cell lines is very similar. The extracts were analysed directly (40 μ l per lane).

MN-related protein from XC cells showed the same pattern as for Rat2-Tk⁻ cells both under reducing and non-reducing conditions, except that its concentration was about 30x lower. The finding of a MN-related protein--p53.5N--in two rat cell lines (Figures 10 and 12) provides the basis for a model system.

None of the other animal cell lines tested contained detectable amounts of MN antigen, even when the highly sensitive immunoblot technique in which the MN antigens are concentrated was used. The MN-negative cells were: Vero cells (African green monkey); mouse L cells; mouse NIH-3T3 cells normal, infected with Moloney leukemia virus, or transformed with Harvey sarcoma virus; GR cells (mouse mammary tumor cells induced with MTV), and NMG cells (normal mouse mammary gland).

Example 8

Radioimmunoassays in Liquid Phase
Using Recombinant MN Protein for
MN-Specific Antibodies and for MN Antigen

5 The genetically engineered MN protein fused with
glutathione S-transferase--GEX-3X-MN--prepared and purified
as described above was labeled with ^{125}I by the chloramine T
method [Hunter (1978)]. The purified protein enabled the
development of a quantitative RIA for MN-specific antibodies
10 as well as for MN antigens. All dilutions of antibodies and
of antigens were prepared in RIPA buffer (1% TRITON X-100
and 0.1% sodium deoxycholate in PBS--phosphate buffered
saline, pH 7.2), to which was added 1% of fetal calf serum
(FCS). Tissue and cell extracts were prepared in RIPA
15 buffer containing 1 mM phenylmethylsulfonylfluoride and 200
trypsin inhibiting units of Trasylol (aprotinin) per ml,
with no FCS. ^{125}I -labeled GEX-3X-MN protein ($2.27 \mu\text{Ci}/\mu\text{g}$ of
TCA-precipitable protein) was before use diluted with RIPA +
1% FCS, and non-specifically binding radioactivity was
20 adsorbed with a suspension of fixed protein A-Staphylococcus
aureus cells (SAC).

In an RIA for MN-specific antibodies, MAb-
containing ascites fluids or test sera were mixed with ^{125}I -
labeled protein and allowed to react in a total volume of 1
25 ml for 2 hours at room temperature. Subsequently, 50 μl of

a 10% suspension of SAC [Kessler, supra] was added and the mixture was incubated for 30 minutes. Finally, the SAC was pelleted, 3x washed with RIPA, and the bound radioactivity was determined on a gamma counter.

5 Titration of antibodies to MN antigen is shown in Figures 11A and 11B. Ascitic fluid from a mouse carrying M75 hybridoma cells (A) is shown to have a 50% end-point at dilution $1:1.4 \times 10^{-6}$. At the same time, ascitic fluids with MAbs specific for MX protein (M16 and M67) showed no
10 precipitation of ^{125}I -labeled GEX-3X-MN even at dilution 1:200 (result not shown). Normal rabbit serum (C) did not significantly precipitate the MN antigen; rabbit anti-MaTu serum (B), obtained after immunization with live MX-infected HeLa cells, precipitated 7% of radioactive MN protein, when
15 diluted 1:200. The rabbit anti-MaTu serum is shown by immunoblot in Example 4 (above) to precipitate both MX and MN proteins.

 Only one out of 180 human sera tested (90 control and 90 sera of patients with breast, ovarian or uterine
20 cervical cancer) showed a significant precipitation of the radioactively labeled MN recombinant protein. That serum--L8--(D) was retested on immunoblot (as in Example 4), but it did not precipitate any p54/58N from MX-infected HeLa cells. Also, six other human sera, including KH (E), were negative

on immunoblot. Thus, the only positive human serum in the RIA, L8, was reactive only with the genetically engineered product, but not with native p54/58N expressed by HeLa cells.

5 In an RIA for MN antigen, the dilution of MAb M75, which in the previous test precipitated 50% of maximum precipitable radioactivity (= dilution $1:1.4 \times 10^{-6}$) was mixed with dilutions of cell extracts and allowed to react for 2 hours. Then, ^{125}I -labeled GEX-3X-MN (25×10^3
10 cpm/tube) was added for another 2 hours. Finally, the radioactivity bound to MAb M75 was precipitated with SAC and washed as above. One hundred percent precipitation (= 0 inhibition) was considered the maximum radioactivity bound by the dilution of MAb used. The concentration of the MN
15 antigen in the tested cell extracts was calculated from an inhibition curve obtained with "cold" GEX-3X-MN, used as the standard (A in Figure 12).

 The reaction of radioactively labeled GEX-3X-MN protein with MAb M75 enabled us to quantitate MN antigen
20 directly in cell extracts. Figure 12 shows that 3 ng of "cold" GEX-3X-MN (A) caused a 50% inhibition of precipitation of "hot" GEX-3X-MN; an equivalent amount of MN antigen is present in 3×10^3 ng of proteins extracted from MaTu-infected HeLa (B) or from Rat2-Tk⁻ cells (C).

Concentrations of MN protein in cell extracts, determined by this RIA, are presented in Table 2 below. It must be understood that the calculated values are not absolute, since MN antigens in cell extracts are of somewhat different sizes, and also since the genetically engineered MN protein is a product containing molecules of varying size.

TABLE 2

Concentration of MN Protein in Cell Extracts

| | Cells | ng MN/mg total protein |
|----|----------------------|------------------------|
| 10 | HeLa + MX | 939.00 |
| 15 | Rat2-Tk ⁻ | 1065.00 |
| | HeLa | 27.50 |
| | XC | 16.40 |
| 20 | T24 | 1.18 |
| | HEF | 0.00 |

The data were calculated from the results shown in Figure 12.

Example 9

Immunoelectron and Scanning Microscopy of
Control and of MX-infected HeLa Cells

As indicated above in Example 1, MN antigen,
5 detected by indirect immunofluorescence with MAb M75, is
located on the surface membranes and in the nuclei of MX-
infected HeLa cells or in HeLa cells grown in dense
cultures. To elucidate more clearly the location of the MN
antigen, immunoelectron microscopy was used wherein MAb M75
10 bound to MN antigen was visualized with immunogold beads.
[Herzog et al., "Colloidal gold labeling for determining
cell surface area," IN: Colloidal Gold, Vol. 3 (Hayat,
M.A., ed.), pp. 139-149 (Academic Press Inc.; San Diego,
CA).]

15 Ultrathin sections of control and of MX-infected
HeLa cells are shown in Figures 13A-13D. Those immuno-
electron micrographs demonstrate the location of MN antigen
in the cells, and in addition, the striking ultrastructural
differences between control and MX-infected HeLa. A control
20 HeLa cell (Figure 13A) is shown to have on its surface very
little MN antigen, as visualised with gold beads. The cell
surface is rather smooth, with only two little protrusions.
No mitochondria can be seen in the cytoplasm. In contrast,
MX-infected HeLa cells (Figures 13B and 13C show the

formation of abundant, dense filamentous protrusions from their surfaces. Most of the MN antigen is located on those filaments, which are decorated with immunogold. The cytoplasm of MX-infected HeLa contains numerous mitochondria (Figure 13C). Figure 13D demonstrates the location of MN antigen in the nucleus: some of the MN antigen is in nucleoplasm (possibly linked to chromatin), but a higher concentration of the MN antigen is in the nucleoli. Again, the surface of normal HeLa (panels A and E of Figure 13) is rather smooth whereas MX-infected HeLa cells have on their surface, numerous filaments and "blebs". Some of the filaments appear to form bridges connecting them to adjacent cells.

It has been noted that in some instances of in vitro transformed cells compared to their normal parent cells that one of the differences is that the surface of normal cells was smooth whereas on the transformed cells were numerous hair-like protrusions [Darnell et al. "Molecular Cell Biology," (2nd edition) Sci. Am. Books; W.H. Freeman and Co., New York (1990)]. Under that criteria MX-infected HeLa cells, as seen in Figure 13F, has a supertransformed appearance.

Further in some tumors, amplification of mitochondria has been described [Bernhard, W., "Handbook of

Molecular Cytology," pp. 687-715, Lima de Faria (ed.), North
Holland Publishing Co.; Amsterdam-London (1972)]. Such
amplification was noted for MX-infected HeLa cells which
stained very intensely with Janus' green, specific for
5 mitochondria whereas control HeLa were only weakly stained.

It should be noted that electron microscopists
were unable to find any structural characteristics specific
for tumor cells.

Example 10

10 Antisense ODNs Inhibit MN Gene Expression

To determine whether both of the p54/58N proteins
were encoded by one gene, the following experiments with
antisense ODNs were performed. Previously sparse-growing
HeLa cells were seeded to obtain an overcrowded culture and
15 incubated for 130 hours either in the absence or in the
presence of two gene-specific ODNs complementary to the 5'
end of MN mRNA. HeLa cells were subcultured at 8×10^5
cells per ml of DMEM with 10% FCS. Simultaneously, ODNs
were added to the media as follows: (A) 29-mer ODN1 (5'
20 CGCCCAGTGGGTCATCTTCCCCAGAAGAG 3' [SEQ. ID. NO.: 3], in 4 μ M
final concentration, (B) 19-mer ODN2 (5' GGAATCCTCCTGCATCCGG
3' [SEQ. ID. NO.: 4] in 4 μ M final concentration and (C)
both ODN1 and ODN2 in 2 μ M final concentration each. (D)

Cells treated in the same way, but incubated without ODNs, served as a control. After 130 hours, extracts from the cells were prepared and analyzed by immunoblotting using ¹²⁵I-labeled MAb M75. Protein extracts from the cells were
5 analyzed by immunoblotting and RIA using MAb M75. Figure 3 provides the immunoblot results of those experiments.

It was found that cultivation of HeLa cells with the ODNs resulted in considerable inhibition of p54/58N synthesis. The 19-mer ODN2 (Figure 3B) in 4 μ M final
10 concentration was very effective; as determined by RIA, it caused 40% inhibition, whereas the 29-mer ODN1 (4 μ M) (Figure 3A) and a combination of the two ODNs (Figure 3C), each in 2 μ M final concentration, were less effective in RIA showing a 25-35% decrease of the MN-related proteins. At
15 the same time, the amount of different HeLa cell protein determined by RIA using specific MAb H460 was in all cell variants approximately the same. Most importantly was that on immunoblot it could be seen that specific inhibition by the ODNs affected both of the p54/58N proteins. Thus, we
20 concluded that the MN gene we cloned coded for both p54/58N proteins in HeLa cells.

The results indicated that the MN twin proteins arise by translation of a single mRNA (consistent with the Northern blotting data). Thus, the twin proteins may

represent either differences in post-translational modification (phosphorylation, protease processing, etc.), or the use of alternative translational initiation sites.

Example 11

5 Northern Blotting of MN mRNA in Tumorigenic and Non-Tumorigenic Cell Lines

Figure 4 shows the results of Northern blotting of MN mRNA in human cell lines. Total RNA was prepared from the following cell lines by the guanidinium thiocyanate-CsCl
10 method: HeLa cells growing in a dense (A) and sparse (B) culture; CGL1 (H/F-N) hybrid cells (C); CGL3 (D) and CGL4 (E) segregants (both H/F-T); and human embryo fibroblasts (F). Fifteen μ g of RNA were separated on a 1.2% formaldehyde gel and blotted onto a Hybond C Super membrane
15 [Amersham]. MN cDNA NotI probe was labeled by random priming [Multiprime DNA labelling system; Amersham]. Hybridization was carried out in the presence of 50% formamide at 42°C, and the final wash was in 0.1% SSPE and 0.1% SDS at 65°C. An RNA ladder (0.24-9.5 kb) [BRL;
20 Bethesda, MD (USA)] was used as a size standard. Membranes were exposed to films at -70°C, with intensifying screens.

Detected was a 1.5 kb MN-specific mRNA only in two tumorigenic segregant clones--CGL3 and CGL4 (H/F-T), but not

in the non-tumorigenic hybrid clone CGL1 (H/F-N) or in normal human fibroblasts. Further, the 1.5 kb mRNA was found in the HeLa cells growing in dense (Figure 4A) but not in sparse (Figure 4B) culture.

5 Thus, the results of the Northern blotting were consistent with other examples in regard to MN-related proteins being associated with tumorigenicity.

Example 12

10 Southern Blotting of Genomic DNAs from Different Vertebrate Species to Detect MN Gene and Restriction Analysis of Genomic DNA of HeLa Cells

Figure 5 illustrates the detection of MN genes in the genomic DNAs of various vertebrates by Southern blotting. Chromosomal DNA digested by PstI was as follows:

15 (A) chicken; (B) bat; (C) rat; (D) mouse; (E) feline; (F) pig; (G) sheep; (H) bovine; (I) monkey; and (J) human HeLa cells. Restriction fragments were separated on a 0.7% agarose gel and alkali blotted onto a Hybond N membrane [Amersham]. The MN cDNA probe labelling and hybridization

20 procedures were the same as for the Northern blotting analyses shown in Figure 4 and described in Example 11. The Southern blot of Figure 5 made with PstI indicates that the MN gene is conserved in a single copy in all vertebrate genomes tested.

HeLa. Further, genomic DNA from HeLa cells was prepared as described by Ausubel et al., Short Protocols in Molecular Biology [Greene Publishing Associates and Wiley-Interscience; New York (1989)], digested with different
5 restriction enzymes, resolved on an agarose gel and transferred to Hybond N+ membrane [Amersham]. The HeLa genomic DNA was cleaved with the following restriction enzymes with the results shown in Figure 17 (wherein the numbers in parentheses after the enzymes indicate the
10 respective lanes in Figure 17): EcoRI (1), EcoRV (2), HindIII (3), KpnI (4), NcoI (5), PstI (6), and PvuII (7), and then analyzed by Southern hybridization under stringent conditions using MN cDNA as a probe.

The prehybridization and hybridization using an MN
15 cDNA probe labelled with ^{32}P -dCTP by random priming [Multi-prime DNA labelling system; Amersham] as well as wash steps were carried out according to Amersham's protocols at high stringency. A 1 kb DNA Ladder [from BRL; Bethesda, MD (USA)] was used as a size standard. Membranes were exposed
20 to films at -70°C , with intensifying screens.

The Southern blotting analysis of HeLa chromosomal DNA showed that the gene coding for MN is present in the human genome in a single copy (Figure 17). The sizes and distribution of MN-positive restriction fragments obtained

using the restriction enzymes KpnI, NcoI and HindIII indicate that the MN gene contains introns, since those enzymes cut the MN genomic sequences despite the absence of their restriction sites in MN cDNA.

5

Example 13

Immunohistochemical Staining of Tissue Specimens

To study and evaluate the tissue distribution range and expression of MN proteins, the monoclonal antibody M75 was used to stain immunohistochemically a variety of human tissue specimens. The primary antibody used in these immunohistochemical staining experiments was the M75 monoclonal antibody. A biotinylated second antibody and streptavidin-peroxidase were used to detect the M75 reactivity in sections of formalin-fixed, paraffin-embedded tissue samples. A commercially available amplification kit, specifically the DAKO LSAB™ kit [DAKO Corp., Carpinteria, CA (USA)] which provides matched, ready made blocking reagent, secondary antibody and streptavidin-horseradish peroxidase was used in these experiments.

20

M75 immunoreactivity was tested according to the methods of this invention in multiple-tissue sections of breast, colon, cervical, lung and normal tissues. Such multiple-tissue sections were cut from paraffin blocks of

tissues called "sausages" that were purchased from the City of Hope [Duarte, CA (USA)]. Combined in such a multiple-tissue section were normal, benign and malignant specimens of a given tissue; for example, about a score of tissue
5 samples of breast cancers from different patients, a similar number of benign breast tissue samples, and normal breast tissue samples would be combined in one such multiple-breast-tissue section. The normal multiple-tissue sections contained only normal tissues from various organs, for
10 example, liver, spleen, lung, kidney, adrenal gland, brain, prostate, pancreas, thyroid, ovary, and testis.

Also screened for MN gene expression were multiple individual specimens from cervical cancers, bladder cancers, renal cell cancers, and head and neck cancers. Such
15 specimens were obtained from U.C. Davis Medical Center in Sacramento, CA and from Dr. Shu Y. Liao [Department of Pathology; St. Joseph Hospital; Orange, CA (USA)].

Controls used in these experiments were the cell lines CGL3 (H/F-T hybrid cells) and CGL1 (H/F-N hybrid
20 cells) which are known to stain respectively, positively and negatively with the M75 monoclonal antibody. The M75 monoclonal antibody was diluted to a 1:5000 dilution wherein the diluent was either PBS [0.05 M phosphate buffered saline

(0.15 M NaCl), pH 7.2-7.4] or PBS containing 1% protease-free BSA as a protein stabilizer.

Immunohistochemical Staining Protocol

The immunohistochemical staining protocol was followed according to the manufacturer's instructions for the DAKO LSAB™ kit. In brief, the sections were dewaxed, rehydrated and blocked to remove non-specific reactivity as well as endogenous peroxidase activity. Each section was then incubated with dilutions of the M75 monoclonal antibody. After the unbound M75 was removed by rinsing the section, the section was sequentially reacted with a biotinylated antimouse IgG antibody and streptavidin conjugated to horseradish peroxidase; a rinsing step was included between those two reactions and after the second reaction. Following the last rinse, the antibody-enzyme complexes were detected by reaction with an insoluble chromogen (diaminobenzidine) and hydrogen peroxide. A positive result was indicated by the formation of an insoluble reddish-brown precipitate at the site of the primary antibody reaction. The sections were then rinsed, counterstained with hematoxylin, dehydrated and cover slipped. Then the sections were examined using standard

light microscopy. The following is an outline of exemplary steps of the immunohistochemical staining protocol.

- | | | |
|----|--|--------------------------|
| 5 | 1. Series of ETOH-baths 100, 100, 95, 95, 70% | 2 min. \pm 1 min. each |
| | 2. dH ₂ O wash - 2x | 2 min. \pm 1 min. each |
| 10 | 3. 3% H ₂ O ₂ as endogenous peroxidase block | 5 min. |
| | 4. PBS wash - 2x | 2 min. \pm 1 min. |
| | 5. normal serum block (1.5% NGS) | 30 min. |
| | 6. primary antibody (Mab M75) | 60 min. \pm 5 min. |
| | 7. PBS wash - 2x | 2 min. \pm 1 min. |
| 15 | 8. biotinylated secondary antibody | 20-30 min. \pm 2 min. |
| | 9. PBS wash - 2x | 2 min. \pm 1 min. |
| | 10. streptavidin-peroxidase reagent | 20-30 min. \pm 2 min. |
| 20 | 11. PBS wash - 2x | 2 min. \pm 1 min. |
| | 12. DAB (150ml Tris, 90 μ l H ₂ O ₂ , 3ml KPL DAB) | 5-6 min. |
| | 13. PBS rinse, dH ₂ O wash | 1-2 min. |
| 25 | 14. Hematoxylin counterstain | 2 min. \pm 1 min. |
| | 15. wash with running tap water until clear | |
| | 16. 0.05% ammonium hydroxide | 20 sec. \pm 10 sec. |
| 30 | 17. dH ₂ O wash - 2x | 3 min. \pm 1 min. |

18. dehydrate 70, 95, 95, 100, 100% 2 min. \pm 1
EtOH min. each
19. xylene 3x 3 min. \pm 1 min.
5 each
20. coverslip with Permount™ [Fisher
Scientific Pittsburgh, PA (USA)]
21. wait 10 min. before viewing results.

Interpretation. A deposit of a reddish brown
10 precipitate over the plasma membrane was taken as evidence
that the M75 antibody had bound to a MN antigen in the
tissue. The known positive control (CGL3) had to be stained
to validate the assay. Section thickness was taken into
consideration to compare staining intensities, as thicker
15 sections produce greater staining intensity independently of
other assay parameters.

The above-described protocol was optimized for
formalin-fixed tissues, but can be used to stain tissues
prepared with other fixatives.

20 Results

Preliminary examination of cervical specimens
showed that 62 of 68 squamous cell carcinoma specimens
(91.2%) stained positively with M75. Additionally, 2 of 6
adenocarcinomas and 2 of 2 adenosquamous cancers of the
25 cervix also stained positively. In early studies, 55.6% (10

of 18) of cervical dysplasias stained positively. A total
of 9 specimens including both cervical dysplasias and
tumors, exhibited some MN expression in normal appearing
areas of the endocervical glandular epithelium, usually at
5 the basal layer. In some specimens, whereas morphologically
normal-looking areas showed expression of MN antigen, areas
exhibiting dysplasia and/or malignancy did not show MN
expression.

M75 positive immunoreactivity was most often
10 localized to the plasma membrane of cells, with the most
apparent stain being present at the junctions between
adjacent cells. Cytoplasmic staining was also evident in
some cells; however, plasma membrane staining was most often
used as the main criterion of positivity.

15 M75 positive cells tended to be near areas showing
keratin differentiation in cervical specimens. In some
specimens, positive staining cells were located in the
center of nests of non-staining cells. Often, there was
very little, if any, obvious morphological difference
20 between staining cells and non-staining cells. In some
specimens, the positive staining cells were associated with
adjacent areas of necrosis.

In most of the squamous cell carcinomas of the
cervix, the M75 immunoreactivity was focal in distribution,

i.e., only certain areas of the specimen stained. Although the distribution of positive reactivity within a given specimen was rather sporadic, the intensity of the reactivity was usually very strong. In most of the
5 adenocarcinomas of the cervix, the staining pattern was more homogeneous, with the majority of the specimen staining positively.

Among the normal tissue samples, intense, positive and specific M75 immunoreactivity was observed only in
10 normal stomach tissues, with diminishing reactivity in the small intestine, appendix and colon. No other normal tissue stained extensively positively for M75. Occasionally, however, foci of intensely staining cells were observed in normal intestine samples (usually at the base of the crypts)
15 or were sometimes seen in morphologically normal appearing areas of the epithelium of cervical specimens exhibiting dysplasia and/or malignancy. In such, normal appearing areas of cervical specimens, positive staining was seen in focal areas of the basal layer of the ectocervical
20 epithelium or in the basal layer of endocervical glandular epithelium. In one normal specimen of human skin, cytoplasmic MN staining was observed in the basal layer. The basal layers of these epithelia are usually areas of proliferation, suggesting the MN expression may be involved

in cellular growth. In a few cervical biopsied specimens, MN positivity was observed in the morphologically normal appearing stratified squamous epithelium, sometimes associated with cells undergoing koilocytic changes.

5 Some colon adenomas (4 of 11) and adenocarcinomas (9 of 15) were positively stained. One normal colon specimen was positive at the base of the crypts. Of 15 colon cancer specimens, 4 adenocarcinomas and 5 metastatic lesions were MN positive. Fewer malignant breast cancers (3
10 of 25) and ovarian cancer specimens (3 of 15) were positively stained. Of 4 head and neck cancers, 3 stained very intensely with M75.

 Although normal stomach tissue was routinely positive, 4 adenocarcinomas of the stomach were MN negative.
15 Of 3 bladder cancer specimens (1 adenocarcinoma, 1 non-papillary transitional cell carcinoma, and 1 squamous cell carcinoma), only the squamous cell carcinoma was MN positive. Approximately 40% (12 of 30) of lung cancer specimens were positive; 2 of 4 undifferentiated carcinomas;
20 3 of 8 adenocarcinomas; 2 of 8 oat cell carcinomas; and, 5 of 10 squamous cell carcinomas. One hundred percent (4 of 4) of the renal cell carcinomas were MN positive.

 In summary, MN antigen, as detected by M75 and immunohistochemistry in the experiments described above, was

shown to be prevalent in tumor cells, most notably in tissues of cervical cancers. MN antigen was also found in some cells of normal tissues, and sometimes in morphologically normal appearing areas of specimens

5 exhibiting dysplasia and/or malignancy. However, MN is not usually extensively expressed in most normal tissues, except for stomach tissues where it is extensively expressed and in the tissues of the lower gastrointestinal tract where it is less extensively expressed. MN expression is most often

10 localized to the cellular plasma membrane of tumor cells and may play a role in intercellular communication or cell adhesion. Representative results of experiments performed as described above are tabulated in Table 3.

TABLE 3

Immunoreactivity of M75 in Various Tissues

| | <u>TISSUE</u> | <u>TYPE</u> | <u>POS/NEG</u> <u>(#pos/#tested)</u> |
|----|--|-------------|---|
| 5 | liver, spleen, lung, kidney, adrenal gland, brain, prostate, pancreas, thyroid, ovary, testis | normal | NEG (all) |
| | skin | normal | POS (in basal layer) (1/1) |
| 10 | stomach | normal | POS |
| | small intestine | normal | POS |
| | colon | normal | POS |
| | breast | normal | NEG (0/10) |
| 15 | cervix | normal | NEG (0/2) |
| | breast | benign | NEG (0/17) |
| | colon | benign | POS (4/11) |
| | cervix | benign | POS (10/18) |
| | breast | malignant | POS (3/25) |
| 20 | colon | malignant | POS (9/15) |
| | ovarian | malignant | POS (3/15) |
| | lung | malignant | POS (12/30) |
| | bladder | malignant | POS (1/3) |
| | head & neck | malignant | POS (3/4) |
| 25 | kidney | malignant | POS (4/4) |
| | stomach | malignant | NEG (0/4) |
| | cervix | malignant | POS (62/68) |

The results recorded in this example indicate that the presence of MN proteins in a tissue sample from a patient may, in general, depending upon the tissue involved, be a marker signaling that a pre-neoplastic or neoplastic process is occurring. Thus, one may conclude from these results that diagnostic/prognostic methods that detect MN

antigen may be particularly useful for screening patient samples for a number of cancers which can thereby be detected at a pre-neoplastic stage or at an early stage prior to obvious morphologic changes associated with
5 dysplasia and/or malignancy being evident or being evident on a widespread basis.

Example 14

Vaccine -- Rat Model

As shown above in Example 7, in some rat tumors,
10 for example, the XC tumor cell line (cells from a rat rhabdomyosarcoma), a rat MN protein, related to human MN, is expressed. Thus a model was afforded to study antitumor immunity induced by experimental MN-based vaccines. The following representative experiments were performed.

15 Nine- to eleven-day-old Wistar rats from several families were randomized, injected intraperitoneally with 0.1 ml of either control rat sera (the C group) or with rat serum against the MN fusion protein GEX-3X-MN (the IM group). Simultaneously both groups were injected
20 subcutaneously with 10^6 XC tumor cells.

Four weeks later, the rats were sacrificed, and their tumors weighed. The results are shown in Figure 14. Each point on the graph represents a tumor from one rat.

The difference between the two groups -- C and IM -- was significant by Mann-Whitney rank test ($U = 84$, $\alpha < 0.025$). The results indicate that the IM group of baby rats developed tumors about one-half the size of the controls, and 5 of the 18 passively immunized rats developed no tumor at all, compared to 1 of 18 controls.

Example 15

Expression of Full-Length MN cDNA in NIH 3T3 Cells

The role of MN in the regulation of cell proliferation was studied by expressing the full-length cDNA in NIH 3T3 cells. That cell line was chosen since it had been used successfully to demonstrate the phenotypic effect of a number of proto-oncogenes [Weinberg, R.A., Cancer Res., 49: 3713 (1989); Hunter, T., Cell, 64: 249 (1991)]. Also, NIH 3T3 cells express no endogenous MN-related protein that is detectable by Mab M75.

The full length MN cDNA was obtained by ligation of the two cDNA clones using the unique BamHI site and subcloned from pBluescript into KpnI-SacI sites of the expression vector pSG5C. pSG5C was kindly provided by Dr. Richard Kettman [Department of Molecular Biology, Faculty of Agricultural Sciences, B-5030 Gembloux, Belgium]. pSG5C was derived from pSG5 [Stratagene] by inserting a polylinker

consisting of a sequence having several neighboring sites for the following restriction enzymes: EcoRI, XhoI, KpnI, BamHI, SacI, 3 times TAG stop codon and BglII.

The recombinant pSG5C-MN plasmid was co-
5 transfected in a 10:1 ratio (10 μ g : 1 μ g) with the pSV2neo plasmid [Southern and Berg, J. Mol. Appl. Genet., 1: 327 (1982)] which contains the neo gene as a selection marker. The co-transfection was carried out by calcium phosphate precipitation method [Mammalian Transfection Kit;
10 Stratagene] into NIH 3T3 cells plated a day before at a density of 1×10^5 per 60 mm dish. As a control, pSV2neo was co-transfected with empty pSG5C.

Transfected cells were cultured in DMEM medium supplemented with 10% FCS and 600 μ g ml⁻¹ of G418 [Gibco
15 BRL] for 14 days. The G418-resistant cells were clonally selected, expanded and analysed for expression of the transfected cDNA by Western blotting using iodinated Mab M75.

For an estimation of cell proliferation, the
20 clonal cell lines were plated in triplicates (2×10^4 cells/well) in 24-well plates and cultivated in DMEM with 10% FCS and 1% FCS, respectively. The medium was changed each day, and the cell number was counted using a hemacytometer.

To determine the DNA synthesis, the cells were plated in triplicate in 96-well plate at a density of 10^4 /well in DMEM with 10% FCS and allowed to attach overnight. Then the cells were labeled with ^3H -thymidine
5 for 24 hours, and the incorporated radioactivity was counted.

For the anchorage-independent growth assay, cells (2×10^4) were suspended in a 0.3% agar in DMEM containing 10% FCS and overlaid onto 0.5% agar medium in 60 mm dish.
10 Colonies grown in soft agar were counted two weeks after plating.

Several clonal cell lines constitutively expressing both 54 and 58 kd forms of MN protein in levels comparable to those found in LCMV-infected HeLa cells were
15 obtained. Selected MN-positive clones and negative control cells (mock-transfected with an empty pSG5C plasmid) were subjected to further analyses directed to the characterization of their phenotype and growth behavior.

The MN-expressing NIH 3T3 cells displayed spindle-
20 shaped morphology, and increased refractility; they were less adherent to the solid support and smaller in size. The control (mock transfected cells) had a flat morphology, similar to parental NIH 3T3 cells. In contrast to the control cells that were aligned and formed a monolayer with

an ordered pattern, the cells expressing MN lost the capacity for growth arrest and grew chaotically on top of one another (Figures 22A-22D). Correspondingly, the MN-expressing cells were able to reach significantly higher
5 (more than 2x) saturation densities (Table 4) and were less dependent on growth factors than the control cells (Figures 22G and 22H).

MN transfectants also showed faster doubling times (by 15%) and enhanced DNA synthesis (by 10%), as determined
10 by the amount of [³H]-thymidine incorporated in comparison to control cells. Finally, NIH 3T3 cells expressing MN protein grew in soft agar. The diameter of colonies grown for 14 days ranged from 0.1 to 0.5 mm (Figure 22f); however, the cloning efficiency of MN transfectants was rather low
15 (2.4%). Although that parameter of NIH 3T3 cells seems to be less affected by MN than by conventional oncogenes, all other data are consistent with the idea that MN plays a role in cell growth control.

Table 4

Growth Properties of NIH 3T3 Cells Expressing MN Protein

| 5 | Transfected DNA | pSG5C/ pSV2neo | pSG5C-MN/ pSV2neo |
|----|--|-------------------|----------------------|
| | Doubling time ^a (hours) | 27.9 ± 0.5 | 24.1 ± 1.3 |
| 10 | Saturation density ^b (cells x 10 ⁴ /cm ²) | 4.9 ± 0.2 | 11.4 ± 0.4 |
| | Cloning efficiency (%) ^c | < 0.01 | 2.4 ± 0.2 |

^aFor calculation of the doubling time, the proliferation
 15 rate of exponentially growing cells was used. ^bThe
 saturation cell density was derived from the cell number 4
 days after reaching confluency. ^cColonies greater than 0.1
 mm in diameter were scored at day 14. Cloning efficiency
 was estimated as a percentage of colonies per number of
 20 cells plated, with correction for cell viability.

Example 16

Acceleration of G1 Transit and Decrease in Mitomycin C
Sensitivity Caused by MN Protein

For the experiments described in this example, the
 25 stable MN transfectants of NIH 3T3 cells generated as
 described in Example 15 were used. Four selected MN-

positive clones and four control mock-transfected clones were either used individually or in pools.

Flow cytometric analyses of asynchronous cell populations. For the results shown in Figures 23A-1 and
5 23A-2, cells that had been grown in dense culture were plated at 1×10^6 cells per 60 mm dish. Four days later, the cells were collected by trypsinization, washed, resuspended in PBS, fixed by dropwise addition of 70% ethanol and stained by propidium iodine solution containing
10 RNase. Analysis was performed by FACStar using DNA cell cycle analysis software [Becton Dickinson; Franklin Lakes, NJ (USA)].

For the analyses shown in Figures 23B-1, 23B-2 and 23C, exponentially growing cells were plated at 5×10^5
15 cells per 60 mm dish and analysed as above 2 days later. Forward light scatter was used for the analysis of relative cell sizes. The data were evaluated using Kolmogorov-Smirnov test [Young, J. Histochem. Cytochem., 25: 935 (1977)]. D is the maximum difference between summation
20 curves derived from histograms. D/s(n) is a value which indicates the similarity of the compared curves (it is close to zero when curves are similar).

The flow cytometric analyses revealed that clonal populations constitutively expressing MN protein showed a

decreased percentage of cells in G1 phase and an increased percentage of cells in G2-M phases. Those differences were more striking in cell populations grown throughout three passages in high density cultures Figures 23A-1 and 23A-2],
5 than in exponentially growing subconfluent cells Figures 23B-1 and 23B-2. That observation supports the idea that MN protein has the capacity to perturb contact inhibition.

Also observed was a decrease in the size of MN expressing cells seen in both exponentially proliferating
10 and high density cultures. It is possible that the MN-mediated acceleration of G1 transit is related to the above-noted shorter doubling time (by about 15%) of exponentially proliferating MN-expressing NIH 3T3 cells. Also, MN
15 expressing cells displayed substantially higher saturation density and lower serum requirements than the control cells. Those facts suggest that MN-transfected cells had the capacity to continue to proliferate despite space
limitations and diminished levels of serum growth factors, whereas the control cells were arrested in G1 phase.

20 Limiting conditions. The proliferation of MN-expressing and control cells was studied both in optimal and limiting conditions. Cells were plated at 2×10^4 per well of 24-well plate in DMEM with 10% FCS. The medium was changed at daily intervals until day 4 when confluency was

reached, and the medium was no longer renewed. Viable cells were counted in a hemacytometer at appropriate times using trypan blue dye exclusion. The numbers of cells were plotted versus time wherein each plot point represents a mean value of triplicate determination.

The results showed that the proliferation of MN expressing and control cells was similar during the first phase when the medium was renewed daily, but that a big difference in the number of viable cells occurred after the medium was not renewed. More than half of the control cells were not able to withstand the unfavorable growth conditions. In contrast, the MN-expressing cells continued to proliferate even when exposed to increasing competition for nutrients and serum growth factors.

Those results were supported also by flow cytometric analysis of serum starved cells grown for two days in medium containing 1% FCS. While 83% of control cells accumulated in G0-G1 phase (S = 5%, G2-M = 12%), expression of MN protein partially reversed the delay in G1 as indicated by cell cycle distribution of MN tranfectants (G0-G1 = 65%, S = 10%, G2-M = 26%). The results of the above-described experiments suggest that MN protein might function to release the G1/S checkpoint and allow cells to proliferate under unfavorable conditions.

MMC. To test that assumption, unfavorable conditions were simulated by treating cells with the DNA damaging drug mitomycin C (MMC) and then following their proliferation and viability. The mechanism of action of MMC is thought to result from its intracellular activation and subsequent DNA alkylation and crosslinking [Yier and Szybalski, Science, 145: 55 (1964)]. Normally, cells respond to DNA damage by arrest of their cell cycle progression to repair defects and prevent acquisition of genomic instability. Large damage is accompanied by marked cytotoxicity. However, many studies [for example, Peters et al., Int. J. Cancer, 54: 450 (1993)] concern the emergence of drug resistant cells both in tumor cell populations and after the introduction of oncogenes into nontransformed cell lines.

The response of MN-transfected NIH 3T3 cells to increasing concentrations of MMC was determined by continuous [³H]-thymidine labeling. Cells were plated in 96-well microtiter plate concentration of 10⁴ per well and incubated overnight in DMEM with 10% FCS to attach. Then the growth medium was replaced with 100 µl of medium containing increasing concentrations of MMC from 1 µl/ml to 32 µg/ml. All the drug concentrations were tested in three replicate wells. After 5 hours of treatment, the MMC was

removed, cells were washed with PBS and fresh growth medium without the drug was added. After overnight recovery, the fractions of cells that were actively participating in proliferation was determined by continuous 24-hr labeling with [³H]-thymidine. The incorporation by the treated cells was compared to that of the control, untreated cells, and the proliferating fractions were considered as a percentage of the control's incorporation.

The viability of the treated cells was estimated three days later by a CellTiter 96 AQ Non-Radioactive Cell Proliferation Assay [Promega] which is based on the bioreduction of methotrexate (MTX) into a water soluble formazan that absorbs light at 490 nm. The percentage of surviving cells was derived from the values of absorbance obtained after subtraction of background.

The control and MN-expressing NIH 3T3 cells showed remarkable differences in their responses to MMC. The sensitivity of the MN-transfected cells appeared considerably lower than the control's in both sections of the above-described experiments. The results suggested that the MN-transfected cells were able to override the negative growth signal mediated by MMC.

ATCC Deposits. The material listed below was deposited with the American Type Culture Collection (ATCC)

at Manassas, Virginia 20110-2209 (USA). The deposits were made under the provisions of the Budapest Treaty on the International Recognition of Deposited Microorganisms for the Purposes of Patent Procedure and Regulations thereunder (Budapest Treaty). Maintenance of a viable culture is assured for thirty years from the date of deposit. The hybridomas and plasmids will be made available by the ATCC under the terms of the Budapest Treaty, and subject to an agreement between the Applicants and the ATCC which assures unrestricted availability of the deposited hybridomas and plasmids to the public upon the granting of patent from the instant application. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any Government in accordance with its patent laws.

| | <u>Hybridoma</u> | <u>Deposit Date</u> | <u>ATCC #</u> |
|----|------------------|---------------------|---------------|
| | VU-M75 | September 17, 1992 | HB 11128 |
| | MN 12.2.2 | June 9, 1994 | HB 11647 |
| 20 | <u>Plasmid</u> | <u>Deposit Date</u> | <u>ATCC #</u> |
| | A4a | June 6, 1995 | 97199 |
| | XE1 | June 6, 1995 | 97200 |
| | XE3 | June 6, 1995 | 97198 |

The description of the foregoing embodiments of the invention have been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teachings. The embodiments were chosen and described in order to explain the principles of the invention and its practical application to enable thereby others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated.

All references cited herein are hereby incorporated by reference.